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CONTRIBUTIONS TO EMBRYOLOGY

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CONTRIBUTIONS TO EMBRYOLOGY, NO. 90.

CULTIVATION OF EMBRYONIC HEART-MUSCLE.

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With four plates.

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THE CULTIVATION OF EMBRYONIC HEART-MUSCLE.

INTRODUCTION.

In cultures of the heart of the embryonic chick six types of cells should be looked for in the outgrowths—heart-muscle, mesenchyme, endothelium, mesothelium, clasmatocytes, and sympathetic nerve-cells and fibers. The present paper is concerned with the general chracteristics of the migrating muscle and its distinction from the mesenchyme, the relation of the muscle-cells to one another (that is, whether or not they form a syncytium), the myofibrillæ, the character of the mitochondria, and the glycogen content of the cells.

The main interest in the past has been the occurrence in the outgrowths of muscle-cells that have become more or less isolated and have shown rhythmic pulsations independent of those exhibited by the explant. Burrows (1910, 1911, 1912a, b) first studied this phenomenon with embryonic chick hearts in plasma media and drew the natural conclusion that it supported in a very emphatic manner the myogenic theory of the origin of the heart-beat. Lake (1916) subsequently confirmed these observations in cultures of very young rabbit hearts in human plasma. It was quite well known before this, however, that "the heart of the chick begins to beat long before any nervous connections with the central system can be established; indeed, the rhythmical pulsation begins at about the stage of 10 somites when the neural crest is yet undifferentiated, and no neuroblasts are to be found anywhere." (Lillie, 1908, p. 259.)

Burrows (1910), in a preliminary report on the cultivation of 60-hour old embryonic chick tissues in plasma, mentions briefly that the muscular elements of the heart grow out much less frequently (in about 3 per cent of the cultures) than the mesenchymal elements, and that they "appear in the form of short chains of striated cells" which "contract rhythmically along that portion of the heart from which they arise." In his more extended account (Burrows, 1911) he states that the muscle-cells generally began to show between the second and the sixth day after transplantation; the cellular activity continued from 6 to 8 days in most of his preparations, and in a few for as long as 12 days. Later (Burrows, 1912a, b) he gave short accounts of the rhythmic activity of a single heart-muscle cell that became isolated from the "cellular syncytia." The rhythmic beat of the "syncytial" outgrowth was similar to that of the explant, while that of an isolated cell was different. He found that rhythmic activity did not occur during the active outwandering of the cells, but later, after the cells became permanently located in a definite position in the clot and were undergoing slow multiplication and differentiation. He also found that the newly grown cells in the continuous-flow type of cultures showed rhythmic activity in 3 out of 15 cultures, while in the ordinary hanging-drop plasma cultures such contractions occurred in only 2 out of 150 cultures. Burrow's figure 2, of muscle outgrowth from the ventricle, looks like mesenchyme or endothelium and was of no help in enabling me to recognize the outgrowths of heart-muscle in cultures. He did not enter into the details of the cytological features of the migrating muscle-cells nor the inter-relationship of these cells, and I am not convinced that he actually followed the differentiation or made any special effort to determine whether the cells form a syncytium or not.

In Congdon's (1915) cultures from the ventricles of chick-embryo hearts in plasma, two types of reticulum appeared—fine and coarse. Neither gave evidence of being separated by cell-walls after Zenker or osmic fixation and iron hematoxylin and erythrosin stain. All intermediate forms of reticulum were encountered but many cultures were predominated by one or the other type. The finer type was more abundant. The coarser growths from the 4-day ventricles he believed to be primitive myocardium. This was rare from 5-day ventricles and entirely absent in older tissue. His figures look like mesenchymal outgrowths from the heart, not like the heart-muscle seen in my plasma and Locke-bouillon-dextrose cultures, so I have serious doubts as to whether he had actual outgrowths of heart-muscle.

Lake (1916) cultivated heart-tissue from young rabbits and rabbit embryos in plasma (3 parts) and distilled water (2 parts). The profusely growing spindle cells resembled fibroblasts. Lake found it difficult to determine whether these cells pulsated independently of the pull of the pulsating explant or not, but in a few cases he saw long cells projecting from the main mass of the explant and pulsating at different rates. From this he concluded that the so-called "fibroblasts" in heart cultures in plasma are contractile and must therefore be considered muscular. This conclusion seems unwarranted. The few projecting cells that did pulsate at different rates were undoubtedly muscle-cells; most of the others, however, were probably either fibroblasts or endothelial cells. I have frequently seen these conditions in plasma and in Locke-bouillon-dextrose cultures and realize how difficult it is sometimes to determine whether cells attached either directly or indirectly to the pulsating explant beat independently or not, unless they have a different rhythm from that of the explant. Most of the migrating cells seen in our plasma or Locke-bouillon-dextrose cultures were, however, either mesenchyme or endothelium.

Levi (1916, 1919) has given the most complete account of the cytological characters of cultures of the embryonic chick heart in plasma. He considers the large, coarse, reticular masses as heart-muscle and as syncytial in structure. He states that the fibrillæ are abundant in the fixed cells and that there are many more smooth than cross-striated ones, and that they appear to pass beyond the limits of a single nuclear field. He emphasizes the amœboid movement of the muscle elements, even though they contain already differentiated myofibrils. He evidently believes that within the embryo the heart-muscle forms a syncytium and that the outgrowth retains for the most part this syncytial condition because of the apparent absence of cell boundaries and the mitotic division of the nuclei without division of the cytoplasm. However, he does recognize that these cells have the potentiality of reacquiring their individuality, as free muscle-cells occur which show lively amœboid movement.

I have puzzled over Levi's two papers and examined and reexamined his figures and I am not convinced that he was dealing with heart-muscle in all cultures. Since his figures are more like the mesenchymal than the muscle outgrowths of our cultures, they were of no help in enabling me to identify heart-muscle in the Locke-bouillon-dextrose or plasma cultures. They are from cultures only 2 to 3 days old and heart muscle does not usually begin to migrate until the second day, according to Burrows, and usually not until the second to the fifth day in my cultures, although short muscle sprouts are occasionally seen by the end of the first day. We have been cultivating cells from explants of the heart for over 10 years and have often gotten, during the first 2 or 3 days, cells similar to those Levi illustrates. We have debated the possibility of those cells which showed such marked fibrillæ on fixation being muscle-cells and, in fact, did sometimes tentatively call them such. It is only comparatively recently that we have obtained cultures with migrating heart-muscle. It was recognized at once as being different from the usual type of migration from the heart. There are places where even now it is difficult to decide on the identity of individual cells, but as a rule the recognition of heart-muscle and its distinction from endothelium, mesothelium, and mesenchyme can be relied upon without question.

I recently examined carefully 7 series of 90 cultures from embryo chick hearts grown in plasma from a young chicken, in order better to compare the plasma cultures with cultures grown in the Locke-bouillon-dextrose medium. All of the 24 cultures from 4-day embryos showed extensive migration of the mesenchyme, which formed a zone from 0.33 to 0.5 mm. wide at the end of the first 24 hours, and from 1.5 to 1.75 mm. at the end of the third day. By the fourth day the outer fringe of migrating cells extended in some cultures 2 mm. from the explant, and on the seventh day 2.5 mm. Owing to the thickness of the zone of migrating cells near the explant, it was sometimes difficult, during the first 2 or 3 days, to determine whether any of the heart-muscle had migrated. In such cultures it often appeared as though everything were migrating away from the explant, muscle as well as mesenchyme, but later one could usually see the clear outline of the muscle part of the explant, which had become more transparent by the departure of many mesenchyme and endothelial cells. Other cultures with thinner explants showed at all times the clear outline of the muscle part of the explant, which was quite markedly different from the mesenchymal outgrowth. It had a yellowish tinge, was more opaque, and underwent rhythmic pulsations which exerted a pull on the surrounding elastic mesenchyme. Frequently cells of the mesenchyme were pulled out by the contraction of the explant and then returned to their former shape as the explant relaxed, creating the impression that the mesenchyme was contracting actively. The muscle outgrowths in this series were similar to those in Locke-bouillon-dextrose medium, to be described later. Of the 24 cultures from 4-day hearts, 8 showed short muscle sprouts at 48 hours, 5 additional cultures had muscle sprouts at the end of the third day, 4 others at the end of the fourth day, and 2 more at the end of the fifth day. Most of these sprouts were very short and often there were only 2 or 3 on the explant. In no case did the muscle outgrowths extend out from the explant over 0.25 mm. The sprouts and outgrowths were quite different from the surrounding mesenchyme. Their cytoplasm was darker and the small round, clear nucleus, with its nucleolus, stood out in marked contrast to the cytoplasm, as compared with that of the mesenchyme cells, which was oval, as a rule large, and difficult to distinguish from the clear cytoplasm. The cytoplasm of the muscle-cells often had a finely longitudinally striated appearance, due to the rich supply of thread-like mitochondria. Sometimes the muscle-cells contracted with the explant, and in one culture an almost isolated cell had a rhythm of its own. The most favorable culture (3-day culture, 4-day embryo), examined with the 1.5 mm. lens, showed an extensive coarse muscle reticulum. The cells contained many long mitochondria which gave the appearance of a longitudinal striation, but no other fibrillæ or cross-striations were visible, yet the cells were beating rhythmically with the explant. Here there was little difficulty in distinguishing the muscle from the mesenchymal reticulum, not only by the pulsations, but by the general character of the cytoplasm, which seemed to be denser than that of the mesenchymal cells, and of the nuclei, which were smaller and round. During the course of the observations the cover-glass was cracked and the culture injured. Shortly afterward cross-striations appeared in both migratory zone and explant, where they had not previously been observed. This, together with other observations, has aroused my suspicions regarding the nature of cross-striations as permanent histological structures.

The other series, from 4, 5, and 7-day hearts in plasma, showed a little muscle outgrowth in a small percentage of the cultures. My plasma cultures did not, on the whole, show either a greater number or a greater extent of muscle migration than the Locke-bouillon-dextrose cultures, and this tends to confirm my belief that Levi, Lake, and others have confused muscle and mesenchyme.

One frequently sees in plasma cultures several days old a sort of spreading of the muscular part of the explant, which has become thinned out by the migration of mesenchyme cells. Such spread-out regions are too thick for detailed cytological observation.

Drew (1922) reports briefly on the growth of heart-muscle from mouse embryos in saline solution plus embryonic juice. "The heart may be grown for generation after generation, all traces of the original fragment having disappeared and the growth consisting of a rapidly growing, pulsating thin sheet of cells." These, with suitable fixation and staining, showed typical myofibrillæ. Drew believes that the cells supposed by others to be fibroblasts are really muscle-cells, as suggested by Lake (1916). I have lately made several series of cultures of the mouse embryo heart in a Drew saline solution plus embryonic extract and find that the heart-muscle migrates out no more frequently or extensively in this than does the embryonic chick heart in the Locke-bouillon-dextrose medium, and that most of the cells that migrate are mesenchyme or endothelium. Drew states that where the heart-muscle cells grow in proximity to connective-tissue cells they show typical myofibrillæ, but if the connective-tissue cells fail to grow or the heart-muscle cells grow away from the connective-tissue cells, they appear merely as spindle-shaped cells and the myofibrils are absent. His figures of isolated heart-muscle cells are not convincing , and even his figure 3a, showing numerous fibrillæ, might be endothelium. We have photographs of endothelial cells from cultures of the liver that are strikingly similar to this figure.

GENERAL CHARACTER OF MIGRATING HEART-MUSCLE.

Over 3,000 cultures were made from hearts of chick embryos varying in age from 3 to 11 days. The majority were from the 6 and 7-day embryos, and of these about 8 per cent showed migrating heart-muscle. The cultures from the 11-day embryos gave about 2 per cent and those from 4-day embryos about 11 per cent muscle outgrowths. Mesenchyme or endothelium appeared in practically all cultures and mesothelium was very common. Occasionally, sympathetic nerve-fibers were encountered and clasmatocytes were not uncommon.

Many different series, of 5 to 50 cultures each, were made during a period of several years. The culture media varied only slightly and consisted of Locke solution 80 to 85 parts, chicken bouillon 15 to 20 parts, and dextrose 0.25 to 1 per cent. There were variations also in the hydrogen-ion concentration. The percentage of muscle outgrowths varied greatly in the different series. In many there was no sign of migrating muscle; some series gave a small, others a fairly large percentage of cultures with extensive migration; still others gave varying numbers of cultures with small outgrowths of muscle. I have not been able as yet to get uniform results with any variation of the Locke-bouillon-dextrose medium, and at times series after series failed to show any sign of muscle migration, to my great discouragement. Consequently, many questions must remain unsolved until a better culture medium can be prepared. I have persisted in the employment of the Locke-bouillon-dextrose medium, because it has answered so well for the study of other types of cells and also because occasionally very fine muscle outgrowths occurred.

The heart-muscle did not usually begin to migrate out until the second day and often not until the fourth or fifth day, or even as late as the eighth, but occasionally short buds were noted at 24 hours. It was often difficult in the living cultures to distinguish the early stages of migrating heart-muscle from the mesenchymal outgrowth. The mesenchyme or endothelium, which began to migrate during the first 24 hours and extended out 2 mm. or more during the next few days, varied greatly in character, and I am not altogether sure that I can distinguish between the two types of cells; therefore, throughout this article the term *mesenchyme* is used, in a very loose sense, for the reticular outgrowth that occurs in practically all the cultures and which may be either mesenchyme or endothelium, or both. The large areas of mesothelium that are so common in my cultures of the heart show all transition stages between the multipolar and the mesothelial type; in fact, one can actually watch the transformation from one to the other under the microscope (W. H. Lewis, 1923). Such mesothelial membranes often show marked fibrillæ in the cells and appear to me to be identical with the membrane figured by Levi (1916, fig. 6), but called by him large *myoblasts*.

The general character of the muscle outgrowths varied somewhat, but these variations did not seem to be dependent upon the age of the embryos (4 to 11 days) from which the hearts were taken nor upon the age of the culture (3 to 9 days). The explants were taken at random and most of the hearts from embryos up to 7 days old inclusive were entirely cut up into small pieces, all of which were used. These explants suffered varying degrees of injury and there were undoubtedly great variations in the diffusion into and out of the explants, depending upon size, form, etc. How far the character of the outgrowth was influenced by this I am unable to determine.

In the living cultures it was usually possible to distinguish the migrating heart-muscle from the mesenchyme by the general character of its outgrowth, which, as a rule, was coarser and thicker than the mesenchyme. The muscle-cells are more refractive and the nuclei are usually round. When rhythmic contractions occur this gives the best criterion, but these were observed in only a small percentage of the outgrowths, and one must be very careful not to confuse the pulls of the contracting explant on the outgrowth of mesenchyme or muscle with independent contractions of the latter. On fixation the cross-striations, when present, serve as positive criteria, but they were rarely present in my series. The presence of smooth fibrillæ in the fixed cells is not sufficient evidence, since both endothelial and mesothelial cells may show them. The presence of glycogen, indicated by the pink color with iodine vapor fixation, is a reliable sign, but, unfortunately, glycogen is not always present and can not be detected in the living. Even with all these criteria, there were cells that defied classification.

The whole question of why cells migrate out from the explant has never been satisfactorily explained. Burrows has suggested that acid degeneration products resulting from cell death within the explant may be an important factor. When cultures are tested by the drop method, used by Lewis and Felton (1922) for the determination of hydrogen-ion concentration of the medium on the cover-glass, one can usually see that the explant is more acid than the surrounding medium. If the center of the explant is more acid than the periphery, there might be some differential action on the two sides of the cell, the inner acid side becoming firmer and contracted as compared with the outer side, thus causing the cell to move outward. The slow migration of the muscle-cells, as compared with the mesenchyme cells, must be due to an intrinsic difference in their cytoplasm.

In some cultures (figs. 1, 2) the rather extensive migration of heart-muscle cells in the form of a reticulum stands out in marked contrast to the looser mesenchyme, which extends far out beyond the muscle reticulum. The culture shown in figure 2 was from the heart of a 7-day embryo. When the culture was 6 days old there was very extensive outgrowth of heart-muscle and both the explant and the muscle reticulum were beating synchronously. This continued, even at room temperature, for over an hour. The outgrowth was examined carefully for fibrillæ and cross-striations, but none was seen. On the ninth day the contractions were less vigorous. The culture was washed with a weak solution of janus green until the mitochondria, which seemed to be mostly granular in form, were colored blue. It was then fixed with iodine vapor. During fixation it was noted that the muscle-cells were rich in glycogen. The fixed specimen contained a few cells with a few cross-striations. The mesenchyme cells, as can be seen in the figure, were contracted, round, and isolated. They were still living but very degenerate, while the muscle-cells, on the other hand, were in fairly good condition, although most of the mitochondria were granular in form. There can be no question that this coarse reticulum consists of heart-muscle. The reticulum is so thick that only at its periphery can one examine the cells satisfactorily with the higher powers.

Figures 4 and 5 show muscle-cells projecting beyond the general edge of the muscle reticulum. They possess the characteristic round nucleus and rather dense cytoplasm with a texture differing from that of the mesenchyme cells. The mitochondria are in the form of granules, rods, and threads. There are no indications of either longitudinal or cross-striations in this particular region, yet I do not hesitate to classify them as heart-muscle cells. Other parts of the periphery (fig. 2) show somewhat similar flattened muscle-cells with round nuclei, granular mitochondria, and absence of striations, and also contracted mesenchyme cells.

In figure 6 there are shown two cells from another part of what appears to be heart-muscle outgrowth in this same culture, but whether they should be classed as muscle-cells or not is rather uncertain. It is possible that they are either mesenchyme or endothelium. On the opposite side of the explant the muscle outgrowth was not so extensive; the cells were flattened and did not show glycogen.

Figure 1 shows a portion of another heart-muscle reticulum from a 5-day culture of the heart of a 4-day embryo. Many cells in this plexus were pulsating and when fixed with iodine most of them showed the deep pink-colored areas characteristic of glycogen. On the opposite side of the explant the muscle migration was much less extensive and in the form of a membrane (fig. 3), somewhat similar to that shown in figures 20, 21, and 22. Since cells in such membranes may pulsate independently of one another and of the cells of the explant, they are evidently to be classed as heart-muscle. The mesenchyme cells formed a loose reticulum which extended far beyond the muscle outgrowth.

The muscle outgrowths were usually not so extensive, as indicated by figures 1 and 2. Figure 7 shows a more common form, in which the muscle-cells extend out as short thick masses closely packed together and flattened out against the cover-glass at their peripheral ends. The mesenchyme immediately about the explant shows in places very marked degenerative changes, a common condition in many heart cultures of this age. Figures 8 and 9 show portions of the muscle outgrowth. The nuclei in most places are back toward the explant in the thicker part of the cells. No contractions were observed either in the explant or in the muscle outgrowth, but I think there can be no doubt that this is heart-muscle. The culture was stained with janus green and fixed with iodine vapor. The mitochondria, forming threads and networks of threads, were exceptionally beautiful in the broad, flat peripheral ends of the cells (figs. 10 to 13). Some of the threads were broken up into a series of rods and granules and most of the others showed a tendency in this direction. Some of them thus appeared like cross-striated fibrillæ (fig. 11), but were not such, as I shall show later on. The cytoplasm displayed a peculiar texture and a tendency to coagulate into extremely fine fibrillæ (figs. 10, 12, 13). The mesenchyme or endothelial cells showed granular mitochondria and marked degeneration (fig. 9). The most common type of muscle outgrowth was that of still shorter projections of the muscle-cells from the edge of the explant. These were usually in the form of pointed processes that did not include the whole cell. They appeared from the second to the fifth or sixth day and rarely increased very much in extent.

Sometimes the muscle-cells migrated out in a very loose reticulum or in small groups of two or more cells that were not easy to differentiate from the mesenchyme or endothelium. Figure 14 shows an outgrowth of this character, and here it was difficult, by the general character of the outgrowth, to make the distinction, but as many of these cells exhibited rhythmic pulsations and (on fixation) fibrillæ (figs. 15, 16, 18, 19) and even cross-striations (fig. 19), their identity as muscle-cells was established. Some, however, were without fibrillæ (fig. 17) and still others, even though they had marked fibrillæ, looked more like endothelial cells (compare with W. H. Lewis, 1922; figs. 16 and 17).

Sometimes the migrating heart-muscle cells formed a more or less continuous sheet or membrane (figs. 3, 20, 21, and 22) in which there was little tendency to overlapping of the cells. When such membranes were treated with silver nitrate the muscle-cells were clearly outlined, as in figures 20, 21, and 22. The muscle-membrane illustrated in figure 21 showed rhythmic contractions and in some places individual cells were beating at different rates. In one region at the edge of the membrane one cell was beating at the rate of 110 per minute, another at the rate of 140 per minute; hence there can be no question that we were dealing with heart-muscle.

IS HEART-MUSCLE A SYNCYTIUM?

It should be clearly understood at the start just what is meant by the term *syncytium.* The definition given by Schafer in Quain's *Anatomy* (1912) is as follows: "When the continuity of the cell substance is complete the congeries of cells receive the name of syncytium." Jordan and Ferguson (1916) state that "tissues in which the cell boundaries are absent are known as syncytia . . . A syncytium may previously arise through nuclear proliferation in the absence of cytoplasmic division, or as the result of the disappearance of original cell boundaries."

At present the generally accepted view of heart-muscle seems to be that of a continuous protoplasmic mass containing nuclei at intervals and with muscle fibrillæ passing through one or more nuclear fields. The older view of separate cells, advocated by Schweigger-Seidel (1869), is founded upon the fact that with nitrate of silver there are distinct indications of transverse septa across the fibers, each intercepted space having, as a rule, a nucleus somewhere near its center. Newer methods, however, have seemed to demonstrate that the myofibrils pass across these septa and are continuous from cell to cell. The generally accepted view of embryonic heart-muscle is also that of a syncytium.

Heidenhain (1899) has described a stage of development in the 3-day duck embryo, in which the heart-muscle has the appearance of a complete syncytium with fibrils, developed in the protoplasm, passing far beyond the bounds of anything that could be considered as individual cells. He has reproduced again in his *Plasma und Zelle* (1911, p. 642) a figure which shows long striated myofibrils passing the territory of more than one nucleus. But the emphasis has been laid on the myofibrils and not on the limitations of the cytoplasm. Heidenhain did not attempt to analyze carefully the cell boundaries. In his sections of fixed and stained material the general appearance was that of fusion, but such material does not lend itself to the determination of the limits of cells that overlap each other to any great extent, and consequently his conclusion that they form a syncytium was unwarranted.

Godlewski (1902), in his principal paper on the development of the heart-muscle in the rabbit embryo, supports the syncytial idea. His material was all fixed, sectioned, and stained, and the evidence for the syncytial nature of the heart-muscle rests solely upon this. His figures undoubtedly represent as nearly as possible what can be made out from sections and they show what appears to be a syncytium. I do not believe, however, that one can determine from such material whether the heart-muscle is a syncytium or an adherent reticulum. If one examine Godlewski's figures with the conception that muscle-cells possess numerous thin processes, sometimes slender, sometimes flat, such as he has shown in his figure 24 and such as we find in the cultures, and tries to imagine the difficulties involved in attempting to determine their limits when the reticulum becomes more compact and the overlapping more extensive, as in his figures 25, 26 and 27, it is evident that final judgment in the matter must remain in abeyance. Godlewski calls attention to the fibrils which appear to pass through several cell territories, but such an appearance is, of course, just as easily explained on the assumption that heart-muscle is an adherent reticulum, with extensive overlapping of cells, as on the assumption that it is a syncytium. We need only picture to ourselves how long, thin processes containing fibrils (see Godlewski's figure 24) would seem to be a part of a neighboring cell to which they were closely applied. The fact that the fibrils are in the periphery of the cells, as Godlewski and others have shown, would tend to help the impression that they were extending into neighboring cells. His figure 28 of a cross-section shows how utterly impossible it would be, from this material, to determine by the fibrils the question of continuity or adhesion. Neither his figures nor his descriptions have convinced me that the heart-muscle with which Godlewski was dealing was a syncytium.

Schlater (1907) finds that the heart in a 2½-day chick embryo (the youngest that he investigated) shows no trace of heart-muscle cells. In his section from the heart of a 7-day embryo no cell borders could be distinguished and in the protoplasmic ground mass or syncytium, penetrated by spaces, lay the free nuclei and myofibrils. He thus concludes that the myocardium in a 7-day embryo is entirely syncytial. Upon examination, however, his figures 1 to 6, plate IV, offer no more evidence for a syncytium that they do for an adherent reticulum; in fact, they seem to me to indicate the latter.

Wieman (1907) considers heart-muscle as made up of a series of cylindrical cells with the fibril bundles running the length of the cells. The demarcation of the adult muscle into cells, however, he regards as rather uncertain. In considering the embryonic differentiation, he treats the heart-muscle as though it consisted of independent cells only, but here again the material was fixed, sectioned and stained.

Mrs. Lewis (1919) studied the development of the cross-striations in the living and fixed whole hearts of very young embryos and stated that "certainly in the embryos of 12 myotomes and older there can be no doubt but that the fibrils passed across several cells, and that the fixed tissue formed a syncytium;" and again, that "the muscle tissue of the heart of the youngest embryo appeared to be more or less a syncytium." But she also stated that in "fixed preparations of the heart-muscle the cell boundaries could seldom be determined." She did not attempt at the time to investigate this aspect carefully and now believes, as I do, that heart-muscle is not a syncytium.

Levi (1919) believes there can be no doubt that the heart-muscle, as it exists normally in the embryo from the second and surely from the third day onward, is a syncytium. He accepts the work of Heidenhain (1899) and Godlewski (1902) as conclusive in this regard, and states (p. 537) that in tissue cultures the syncytial structure is modified more or less in the zone of invasion (outgrowth zone) by the active movements of single portions of the syncytium. When the protoplasmic movements are limited, the typical syncytial constitution of the protoplasm is repeated in the outgrowth, as shown in his figure 5. When the movements become active and the myoblasts are exposed to the coagulum, the cells become free and the original architecture disappears; this is independent of the degree of development of the tissue in the explant (see his figure 34). All this demonstrates, according to Levi, that, even if the cellular individuality seems to be lost in the myocardial syncytium, there remains in them, in spite of this, the potentiality of reacquiring their individuality. Since I am not convinced that Levi was dealing in all cases with outgrowths of heart-muscle, his views add nothing to the evidence one way or the other.

While the evidence from the literature, such as it is, supports the idea that embryonic heart-muscle is a syncytium, I find from personal communications with histologists here and there that many of them have their doubts about the correctness of this view and realize that the syncytial conception of heart-muscle is part of the more or less popular or general idea, which has been held from time to time, that many tissues of the body are syncytial in nature.

I have already mentioned that the arrangement of the heart-muscle varied considerably in the outgrowths. In many of the cultures the cells were joined together in the form of a loose reticulum in which it was usually impossible to determine whether cell boundaries existed or not (figs. 1, 2). In such reticuli it is beyond the power of any one to decide between an overlapping of cells and fusion, and while the general appearance is that of fusion in both living and fixed specimens, the final decision can not be made from such evidence. In places at the periphery muscle-cells may become almost separated from the general reticulum (figs. 4, 14, 15, 17, 18), but it is often impossible to determine between fusion and adhesion at some point in the living and in the fixed preparations. I have, however, even in the living cultures, occasionally been able to follow the entire cell boundary and to demonstrate it to others.

In the rare cases where rhythmic pulsations of the muscle-cells of such outgrowths were observed, individual cells in the reticulum, or what appeared to be individual cells, were beating at a different rate from the explant and sometimes even at a different rate from nearby cells. In a 5-day culture from a 7-day embryo with quite an extensive outgrowth of muscle, the explant was pulsating regularly at the rate of 32 beats a minute. What appeared to be a single cell forming part of the reticular outgrowth was beating somewhat irregularly at the rate of 144 beats per minute and, not far away, another cell was pulsating at the rate of 100 beats per minute. No other parts of the reticulum were seen to pulsate. The explant 24 hours later was beating somewhat irregularly at the rate of 16 per minute, while the rate of the first cell had reduced from 144 on the previous day to 64 per minute, with a somewhat irregular rhythm. The other cell did not show pulsations. On the next day the explant was beating more slowly, irregularly, and at times intermittently, but at a different rate from the first cell, which was beating irregularly at the rate of 35 per minute. The second cell did not pulsate. The cells forming the muscle outgrowth were all living during this period and it seemed highly improbable that one or two cells could pulsate and the neighboring cells remain quiescent if they were connected by fusion. While such evidence is not conclusive, it points to adhesion rather than to fusion.

In a 7-day culture from a 4-day embryo a loose reticulum was observed in which were numerous muscle-cells beating at different rates (fig. 15). All the cells shown in this figure are muscle-cells; most of them were beating rhythmically but at different rates. Two of the cells shown at a higher magnification in figure 16 appear to be fused, but an intercellular boundary can be seen (*a*) partly separating the two cells except in one region (*b*), where a long thin process from the cell at the left is evidently continued on to the cell at the right, with no visible line of demarcation, and the fibrils appear to pass from one cell to the other. This, I believe, is only an illusion, due to the impossibility of seeing the limits of thin, overlapping edges. The important fact to be emphasized just here is that these two cells were beating rhythmically at quite different rates and continued to do so while under observation. While it is possible, it seems hardly probable that they would have pulsated at different rates if there had been actual fusion at any place.

Sometimes cells on the edge of a pulsating muscle outgrowth were observed to beat at rates different from it and from each other. Figure 22 shows a heart-muscle membrane that was beating rhythmically at a different rate from that of some of the projecting cells. Burrows (1910) apparently did not observe in plasma cultures independent pulsations of the muscle-cells forming part of the outgrowth reticulum. He states that the muscle elements "appear in the form of short chains of striated cells" which "contract rhythmically along with that portion of the heart from which they arise." Lake (1916), who cultivated the heart-tissue from fetal and young rabbits, saw, in a very few cases, long cells, which he considered muscle, projecting from the main mass and pulsating at different rates.

The heart-muscle cells that migrate out on the cover-glass have a great tendency to stick together, and it is rather rare for individual cells to migrate away from the outgrowth and become entirely isolated from the muscle reticulum. Burrows (1912a) speaks of the rhythmic activity of isolated single heart-muscle cells, in plasma cultures of chick embryo hearts, which become separated from the "cellular syncytia." He did not investigate especially the nature of the general heart-muscle outgrowth, but assumed, as we have done in the past, that it was syncytial in nature and in accord with the usual conception of the muscle, as found in the embryo.

Heart-muscle sometimes migrates out as a flat membrane (figs. 20–22), which may or may not show rhythmic contractions. When such membranes are treated with silver nitrate, characteristic silver markings are found separating the individual cells, indicating very clearly that such membranes are not syncytial in nature. The more complicated reticular outgrowths were not treated with silver nitrate, for it seemed hardly possible that this method would help in such examples.

In places where the muscle outgrowth is mixed with fairly normal endothelium or mesenchyme, there are often connecting processes between muscle and mesenchyme that appear just as much fused as those between muscle-cells, yet the possibility of the former being a real fusion would scarcely be accepted by any one, in spite of the fact that the eye could detect no line of separation.

The evidence, then, from fixed and sectioned embryonic hearts does not show in a satisfactory manner the relations of the heart-muscle cells, while that from tissue cultures indicates at times that it consists of individual cells. In many places in the cultures it is impossible to determine whether there is fusion or adhesion, but the partial or occasionally complete isolation of individual cells, the independence of rhythmic contractions, and the silver-nitrate picture, all tend to show that heart-muscle cells are adherent and not fused. A study of the behavior of mesenchyme cells in cultures led to a similar conclusion in regard to these cells (W. H. Lewis, 1919).

MYOFIBRILLÆ AND CROSS-STRIATIONS.

Although many observations were made with both bright and dark fields on living muscle-cells in the cultures, fibrillæ were rarely seen in them, yet when such fibrillæ-free cells were afterwards fixed with iodine vapor, fibrils often, though not always, appeared. It was possible to watch the gradual appearance of the fibrils during the fixation. Since they are so rarely seen in the living cells, even in those that pulsate, it is not at all improbable that the fibrils occurring in fixed cells are fixation artifacts, dependent upon the peculiar manner in which the contractile cytoplasm coagulates. If this is true, then the contractile substance probably possesses a definite polarity which is molecular in nature. This polarity is also clearly indicated by the very phenomenon of contraction, which always takes place in a definite direction. *Contractile substance* and *contractile cytoplasm* are here used synonymously and are considered, for the present at least, as being part of the living cell. Even when cells are not contracting they are usually under tension, the direction of which no doubt corresponds to the polarity of the contractile substance, and it is probably along such lines of tension that the fibrils appear. The tension exerted on the coagulating contractile substance would seem, then, to determine the position of the fibrils in fixed muscle-cells, just as it doubtless does in other types of cells that are fixed under tension, such as smooth muscle, mesothelium and endothelium.

The idea that the myofibrillæ are not true histological units is not new and has been advanced by Heidenhain (1899, 1911) and others. We find in the fixed muscle-cells of cultures the same variation in the size of the fibrillæ that Heidenhain lays stress upon as indicating lack of a true structure. Since the visible fibrillæ vary down to the limits of visibility, it is not unlikely, as Heidenhain suggests, that there are ultramicroscopic ones that vary in size down to the contractile molecule, or molecular aggregate. Although Heidenhain evidently arrived at his conclusions from the study of fixed material only, he apparently believes that the fibrillæ exist as such in the living cells.

Even though we consider the myofibrillæ as fixation artifacts, they probably give us some indication of the condition of the contractile substance—the amount present, the degree of its differentiation, and the tension exerted by it or on it during fixation. The presence of the contractile substance gives to the living muscle-cell a slightly different appearance from that of an ordinary mesenchymal or endothelial cell. The living muscle-cells seem to be somewhat darker, as though the cytoplasm were denser. There is, of course, the possibility that fibrillæ may be present though not visible, but there is as yet no way of proving this.

The fixed muscle-cells showed great variation in the number and size of the fibrillæ. Sometimes they were numerous and prominent (figs. 15, 16, 18, 19); in the majority of the cultures, however, they were only slightly developed (figs. 10–13). When no distinct fibrillæ were present (figs. 4, 5, 17) the cytoplasm usually had a peculiar texture, as though it were denser than that of an ordinary mesenchyme cell. Such cells when fixed often seemed to have an extremely fine striation, much more delicate than that usually ascribed to the finest myofibrils. These cells, when living, sometimes exhibited rhythmic contractions (fig. 17), indicating that contraction is not always dependent upon something in the cell which, on fixation, forms myofibrils. These variations in the number and size of the fibrillæ probably indicate differences in the amount of contractile cytoplasm, in the state of its differentiation, and in the tension. The amount of the contractile cytoplasm and the state of its differentiation depend upon the extent of either a progressive differentiation or a progressive dedifferentiation.

As already mentioned, I have in a few instances been able to see what might be considered as definite myofibrillæ in living cells, and, in some instances, the same fibrils were recognized after fixation with iodine vapor. This does not, however, convince me that such structures are permanent cell organs. The same appearance I have noted more frequently in mesothelial and endothelial cells. Here the fibrillæ may come and go, depending for their position, I believe, upon some pull exerted upon the cytoplasm. The direction of the fibrillæ usually coincides with the direction of pointed pseudopodia, out into which they frequently run. Such pseudopodia seem to be under considerable tension, as evidenced by their rapid retraction when unduly disturbed. It is quite possible that the tension or pull extends back across the cell-body in the same line and thereby produces a temporary gelation of the ectoplasm into a visible fibril along the path of the pull. When a pseudopodium retracts and the tension is released, the gelation is reversed, the fibril disappears, and the cytoplasm once more becomes homogeneous. The idea that tension can produce a gelation of cytoplasm that is reversible is of fundamental importance in muscle physiology. The explanation of the increase in the number and size of the fibrillæ in our fixed specimens is that the fixation itself produces first an increased tension with consequent gelation in the direction or directions determined by the polarity of the contractile material and the position of the fixed borders of the cell. In smooth-muscle cells, where we have had more material for observing these fibrillæ than in heart-muscle, the relation of the fibrillæ to the lines of pull of the pseudopodia is often very convincing.

Fibrillæ may extend in two or even three directions, depending upon the directions in which the cell is being pulled out by the pointed pseudopodia. Sometimes there are many small pointed pseudopodia along one edge of a cell with many corresponding fibrillæ. The mesothelial cells, especially those found in cultures of the heart, often possess numerous parallel fibrillæ that can be traced into the fine pointed pseudopodia that overlap neighboring cells in a membrane. Levi (1916, fig. 6) has pictured such a membrane, although he erroneously designates it as being composed of large myoblasts with polygonal contour uniting to form a membrane. I think he was misled by the presence of the fibrillæ, considering the latter as diagnostic of muscle. They are certainly not diagnostic of muscle, as they are also abundant in endothelial cells from cultures of the liver (Lewis, 1922). Levi's figures 28 to 31, which he calls myoblasts and which are richly supplied with fibrillæ, appear to me, judging from my experience with heart cultures, to be mesothelial cells and not muscle. Although I have very great admiration for Levi's numerous contributions, I have been unable to reconcile most of his figures of myoblasts with those that I have observed. His myoblastic syncytium of skeletal muscle, shown in his figure 26, is also unlike any cultures of skeletal muscle that I have seen. He worked, for the most part, with younger embryos than I have, and that may in part explain the great differences. His figures 33 and 35, however, showing myoblasts from the heart of an 8-day embryo, resemble in general the muscle outgrowth in our cultures from 6 and 7-day hearts. The fibrillæ observed by him (Levi, 1916, 1919) in living cells were probably of the same nature as the tension fibrils described above and may account for his confusion of endothelium or mesothelium with muscle.

In fixed preparations I found cross-striated fibrils to be exceedingly rare among the migrating cells (figs. 18, 19). The most pronounced examples showed the typical Z, Q and J bands. This condition was more often observed in cells near the explant than among those that had migrated some distance away. Occasionally cells were seen with cross-striated fibrillæ showing only a wide dark segment and a narrow light band. No cross-striations were seen in the living cells, but this may have been due to the comparative rarity of the cross-striations in cultures. According to Mrs. Lewis (1919), the cross-striations are already present in fixed heart-muscle of the 10-somite (28 to 30 hours' incubation) chick embryo. After the fourth day all the muscle-cells in the normal heart are supposed to show, on fixation, a considerable number of cross-striated fibrillæ. It is possible that the differentiation within the normal heart may proceed more rapidly in some cells than in others, but, even so, the bulk of the cells in the hearts of 4 to 11-day embryos undoubtedly show cross-striated fibrillæ on fixation.

How, then, are we to account for such great differences in the condition of the fibrillæ in the fixed migrant cells? Since most of the migrant cells showed less differentiation than the cells in the hearts from which the explants were taken, it seems obvious that the cells, either in the explant or during migration, must have undergone a retrogression or dedifferentiation, unless we assume that the heart contains many muscle-cells less differentiated than those hitherto recognized in ordinary sections and spreads. If this be the case, it might be argued that these cells would be the ones most likely to possess migratory powers. Some of the small hearts were cut into 20 or 30 pieces, which were used as explants. Occasionally half a dozen of these would give rise to migrating muscle-cells, indicating that the less differentiated muscle-cells, if there were any, must have been scattered through the heart-wall more or less in patches of varying size, to correspond to the extent of the outgrowths, from a few to a great multitude of cells. These outgrowths are sometimes too extensive (figs. 1, 2), however, to be accounted for as having arisen from patches of more or less undifferentiated cells. Until we have some evidence, from sectioned material, of the existence of such undifferentiated patches in normal hearts from embryos of 4 days of incubation and up, I shall assume that dedifferentiation has taken place. Most of this dedifferentiation must occur within the explant during the 2 to 6 days usually elapsing before the muscle-cells begin to migrate, because the cells that appeared first at the edge of the explant were less differentiated than the cells within the explant at the time the culture was made.

Skeletal-muscle fibers show the same loss of myofibrillæ and cross-striation when migrating in cultures (Lewis and Lewis, 1917).

Burrows (1912a) mentions the differentiation of heart-cells that have come to rest after migrating out from explants of very young hearts. Although he does not go into details, it seems possible that cells from hearts younger than those we have used may possess the ability to differentiate after they migrate out. Shipley (1916) found in very early stages that when that part of the embryonic area which includes the heart primordium was put into a plasma drop, heart-muscle developed in the explant and began to beat rhythmically after an incubation period of 24 to 30 hours. Differentiation within the explant is possible for other organs, such as the metanophros, as shown by Rienhoff (1922), but impossible in the outgrowth unless the conditions are suitable, as when the mesenchymal outgrowth is excessive.

In the normal heart the process of differentiation from the primitive myoblast to the fully cross-striated cell is supposedly a gradual one with numerous intermediate stages, both as regards the nature of the chemical composition of the contractile substance and the amount of it in the cell. The best indication we have of this is the condition of the cytoplasm after fixation. The various stages, as indicated by fixed material, can be arranged somewhat as follows: (1) dense, rather opaque cytoplasm, (2) slight indication of striation and of rows of granules (Godlewski); (3) smooth fibrillæ; (4) simple cross-striations (light and dark bands on the fibrils), and (5) the complex cross-striated fibrils with the Z, Q and J bands. These are the coagulation pictures of changes and, as I have already indicated, do not represent actual structures in the living cell. The fixed migrating cells in the cultures show all these stages. Figure 19, from the most highly differentiated cells observed in any of the cultures, contains fibrils with the Z, Q and J bands. The two fibers or bundles of fibers with the most marked cross-striations show a gradual fading out of the latter toward either end. The Z bands seem to go first and some fibers show only the wide dark band Q and the narrow light band J. The mitochondria, which are grouped around the nucleus, have no special relation to the fibrils.

In the thin, expanded end of the muscle-cell shown in figure 10 there are many slight parallel striations that have no particular relation to the rather complicated mitochondrial net. A somewhat similar condition is shown in figure 12. In figure 11 the mitochondria are broken up into short disconnected rods, and although they appear somewhat like cross-striated fibrils they have nothing in common with them. Such segmented mitochondria are not uncommon in mesenchyme and endothelial cells. The cell shown in figure 5, from a 9-day culture of the heart of a 7-day embryo shows the rather dense cytoplasm but no trace of fibrils or rows of granules. The scattered mitochondria show various forms. There is no indication that the mitochondria have anything to do with the so-called myofibrils.

It was not possible to tell, by the condition of the fibrils in the culture cells, whether the explants were from 4 or 7-day embryos, or whether the cultures were 6 or 9 days old. The most highly differentiated cells were found, for example, in a 7-day culture from the heart of a 4-day embryo (fig. 19), while cells from a 9-day culture of the heart of a 7-day embryo showed no trace of fibrils. If the conditions in these cells represent various stages in dedifferentiation, then it seems very probable that the process of dedifferentiation is very much like the reverse of differentiation. There is, of course, another possibility—namely, that the cells may have first dedifferentiated within the explant and subsequently redifferentiated after migrating out some distance. We have not been able to follow such changes in living cells, and so have no direct method for determining this.

One can not always distinguish muscle from endothelium by the presence of fibrillæ alone, as I have already noted. The fact that endothelial cells often show, on fixation, numerous fibrillæ very much like the smooth fibrillæ of muscle explains how Levi might have mistaken them for heart-muscle.

The appearance of continuity of the coagulation fibrils from cell to cell in fixed material is probably not due to actual continuity of the fibrils but to the fact that these cells overlap one another and that the fibrillæ are very close to the surface, often extending out to the very ends of even very thin processes, which may overlap several cells. Then again, the direction of the tension in such adjoining muscle-cells is more or less the same and the fibrils would tend to form continuously along the same line.

Since myofibrils are artifacts or transitory structures, and since muscle-cells in which no fibrils or cross-striations can be detected, either before or after fixation, beat rhythmically, theories of muscle contraction can not be based upon visible fibrils or cross-striations with any assurance of possible validity. The conditions which are found in cultures practically force us to base our theories on the physical and chemical conditions of the contractile molecule itself. The configuration of such gigantic colloidal molecules may well be that of elongated flexible crystals or threads arranged with their long axes parallel to the direction in which contraction takes place. Contraction then might result from the shortening and broadening of these unstable molecules by either physical or chemical alterations in their structure.

GLYCOGEN.

Glycogen, according to Allen (1919), is found more abundantly in the heart than in any other organ of the chick embryo. It begins to appear there at about 28 hours of incubation and is abundantly present at 4, 6 and 10 days. Various other tissues, yolk-sac, ectoderm, brain, spinal cord, pharynx, intestine, skeletal muscle and cartilage, also contain glycogen at various ages (Gage, 1917; Allen, 1919). It is not surprising then, that the iodine fixation should reveal the presence of glycogen in many of the cultures of heart-muscle.

When the cultures were fixed with iodine vapor there often appeared, within a minute or so before the cells acquired the yellow iodine tint, areas of diffuse pink in the muscle-cells. Sometimes each cell had one to several such areas, as large or larger than the nucleus, and globular, elongated or irregular in shape. Their location was variable and there seemed to be no region of predilection. Sometimes the areas were near the nucleus, sometimes near the periphery, or out in one of the large processes. Only occasionally did pink areas seem to be distinctly vacuolar-like, in that a sharply defined wall surrounded them. These areas seemed to be of very fluid consistency as though the glycogen were in solution. Shortly after the pink color appeared in the muscle-cells, globules of pink fluid began to appear outside the cells, increasing in size as the pink areas within the cells decreased in color. These extruded globules were sharply outlined by a very thin wall from the surrounding culture fluid. Not all the pink areas within the cells diffused out in the form of globules. Before the pink color appeared in these glycogen areas it was not possible to detect them, but after the color had disappeared from the cell they could sometimes still be recognized for a while as clear homogeneous areas.

This reaction of heart-muscle cells to iodine corresponds in almost every particular to the reaction of certain cells in cultures of tissues from the fish embryo, observed by Mrs. Lewis (1921), except that she describes the glycogen areas as forming blebs, indicating that they seemed to be attached to the cells, while in the heart-muscle the extruded globules lost all connection with the cells. The glycogen appeared to be in solution, but whether it was localized in the areas that colored pink with iodine before the cells were thus treated I could not determine. It may be that the glycogen was more or less diffused throughout the cytoplasm before the application of the iodine. There was no indication that the glycogen was in a granular form. These glycogen areas were noted in 5- to 11-day cultures of hearts of 4- to 7-day chick embryos.

Since in some cultures there was no indication of glycogen, I am inclined to believe that the cells in the cultures possess little or no power of storing the dextrose from the medium as glycogen. In those cells in which the heart-muscle showed no signs of glycogen, we must assume that the glycogen had either diffused out of the cells or had been used up by them. In some of the series almost every culture with migrating heart-muscle showed glycogen, while in other series the reverse was true, indicating that slight differences in the media were responsible in part for the variations in this respect. Neither the age of the embryo, the age of the culture, nor the contractile activity of the explant or outgrowth seemed to influence the amount of glycogen. We have no data concerning any variation in the hearts themselves before the explants were made.

Heart-muscle is the only tissue so far noted in cultures from chick embryos that contains glycogen. The other tissues, like many of the heart cultures, must have lost or utilized their glycogen before or as they migrated out from the explant. The presence of glycogen is an important criterion for the identification of heart-muscle in cultures in Locke-bouillon media, but not an infallible one, since it is not always present.

SUMMARY.

1. In the Locke-bouillon-dextrose medium, heart-muscle cells from embryonic chicks migrate out from the explant along the cover-glass in the form of a reticulum or membrane in about 8 per cent of the cultures.

2. Such outgrowths of heart-muscle form adherent reticuli and not syncytia. This is based on the following facts:

   (*a*) Individual cells often beat at a rhythm of their own, different from that of neighboring cells in the reticulum.

   (*b*) Single cells may migrate and become completely separated from the reticulum.

   (*c*) Distinct cell boundaries are sometimes visible, especially when the membranous-like outgrowths are treated with silver nitrate.

3. It thus seems probable that in the normal heart the cells are adherent and not fused into a syncytium.

4. Preceding or during their migration, heart-muscle cells appear to undergo retrogressive changes and on fixation do not contain as many fibrils or cross-striations as those in the normal heart of the same age.

5. Fibrillæ are not true cytological structures. Those occasionally seen in living cells are probably the result of gelation (due to tension), which is reversible. Those seen in fixed material are dependent upon the peculiar manner in which the contractile substance coagulates.

6. Since rhythmic contractions take place in cells that are free from fibrils and cross-striations, theories of contraction must be based on changes in the contractile molecule.

7. The mitochondria vary in different cells of different cultures, from threads and plexuses of threads to rods and granules, their shape probably depending upon the metabolic condition of the cell. They do not appear to have any relation to the myofibrils.

8. Glycogen, in large homogeneous patches, is frequently seen in the muscle-cells as they are fixed with iodine vapor.

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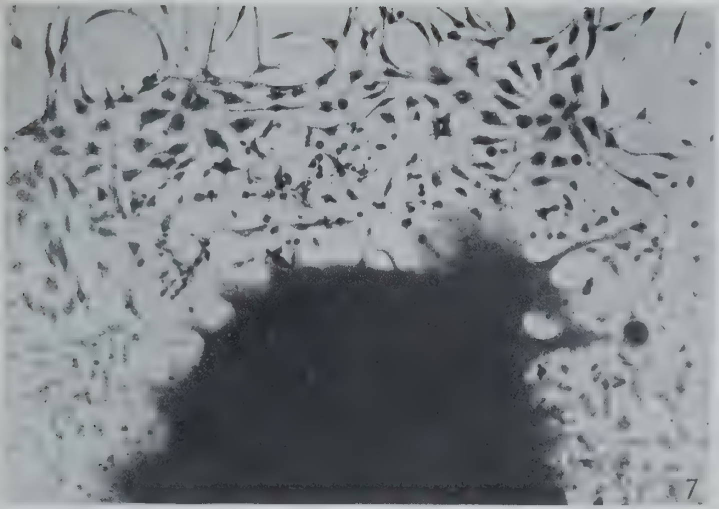

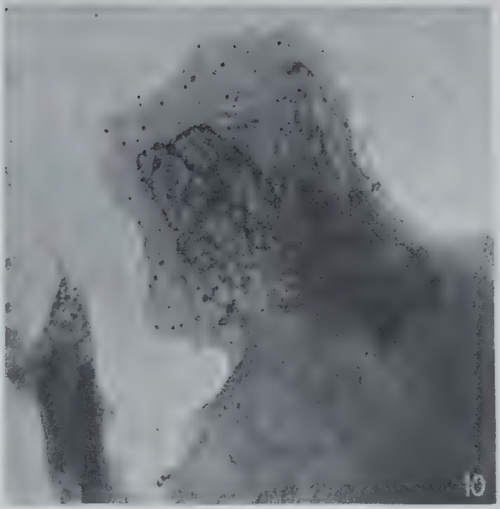

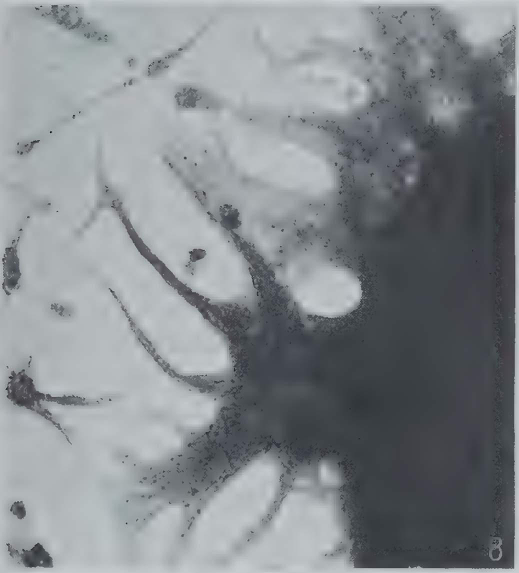

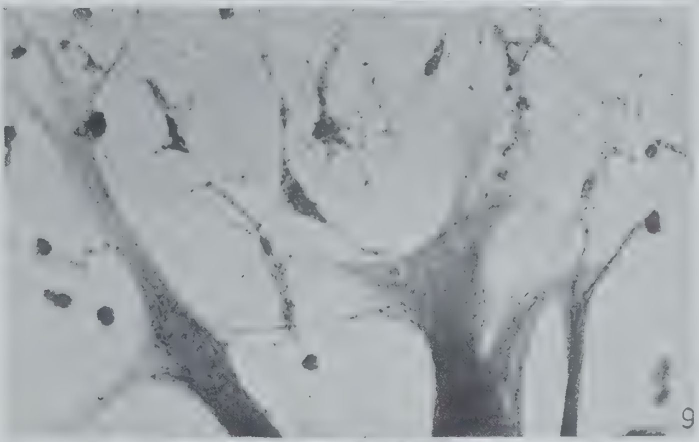

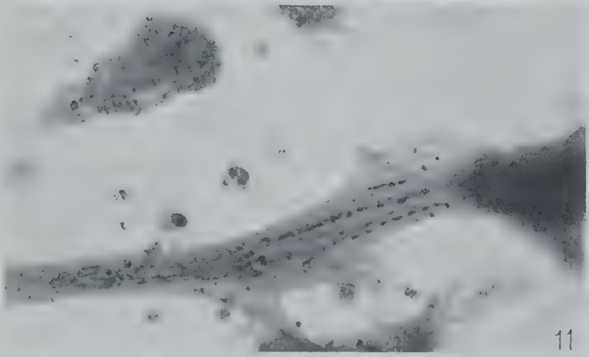

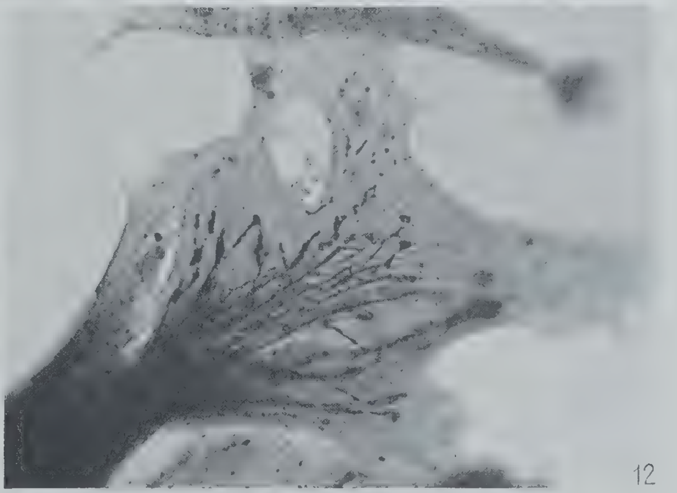

FIG. 7. Culture 752. 6-day culture from heart of a 7-day embryo. Muscle (*mu*) outgrowths are short, flat, and overlapping. Inner zone of mesenchyme (*me*) is very degenerate. Janus green, iodine. ×117.

FIG. 8. Culture 752. Portion of muscle (*mu*) outgrowth and degenerate mesenchyme cells (*me*). ×384.

FIG. 9. Culture 752. Portion of muscle (*mu*) outgrowth and degenerate mesenchyme (*me*). ×384.

FIG. 10. Culture 752. Tip of flat muscle outgrowth with mitochondrial net and slight indication of fibrillæ. ×1160.

FIG. 11. Culture 752. Tip of muscle outgrowth with segmented mitochondria; very slight indication of fibrils. Degenerate mesenchyme cell (*me*). ×1160.

FIG. 12. Culture 752. Tip of flat muscle outgrowth with abundant mitochondria. Slight indication of fibrils at right angles to mitochondria at *f*. ×1160.

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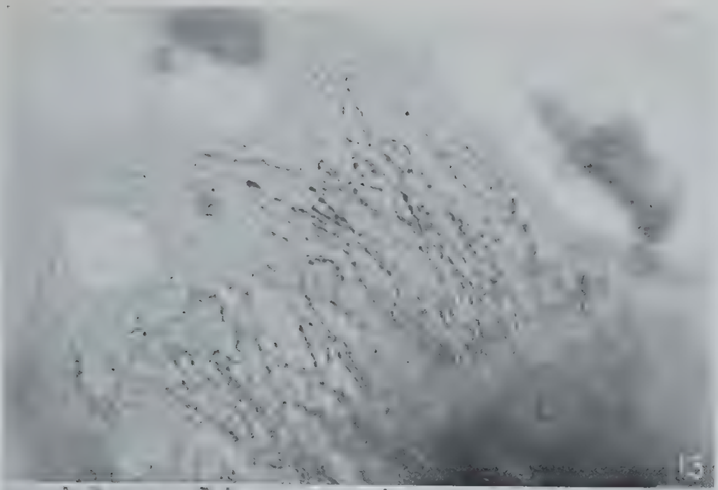

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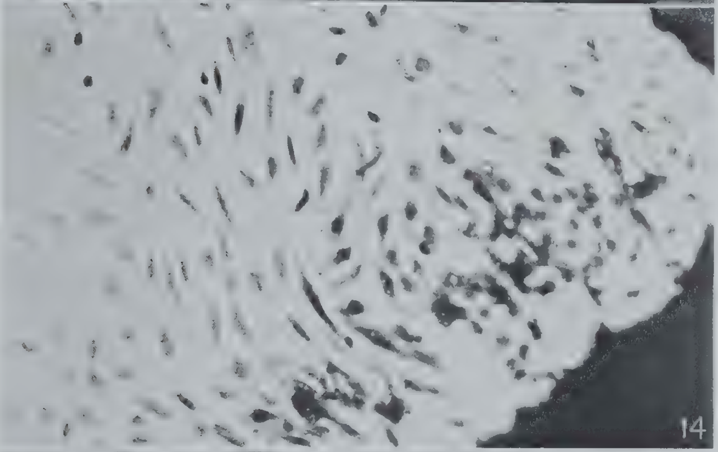

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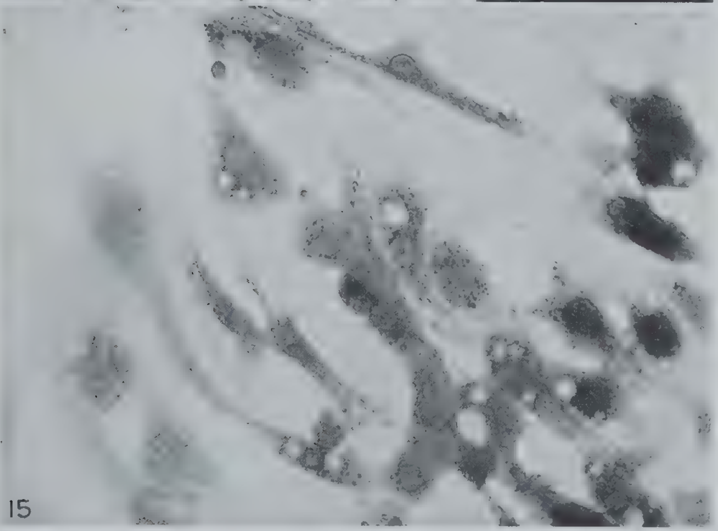

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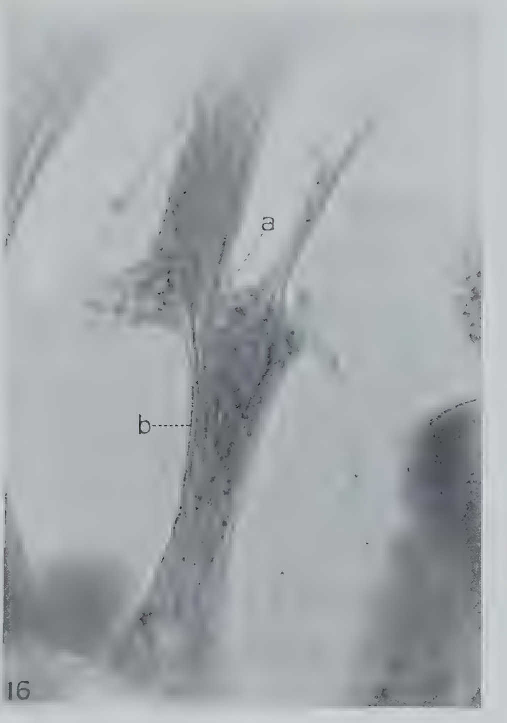

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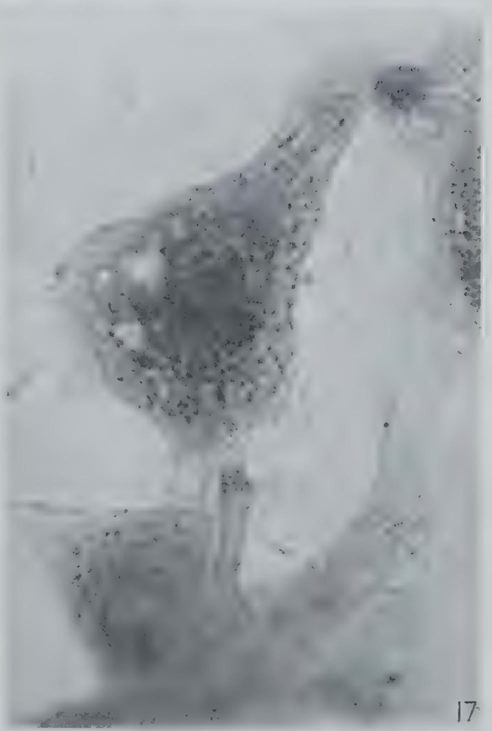

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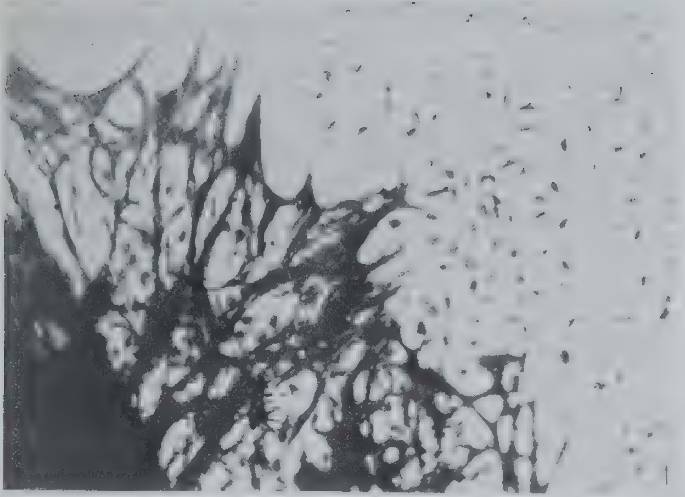
Fig. 13. Culture 752. Tip of flat muscle outgrowth; long, partially segmented mitochondria. The cytoplasm shows slight indication of fibrillation. ×1160.

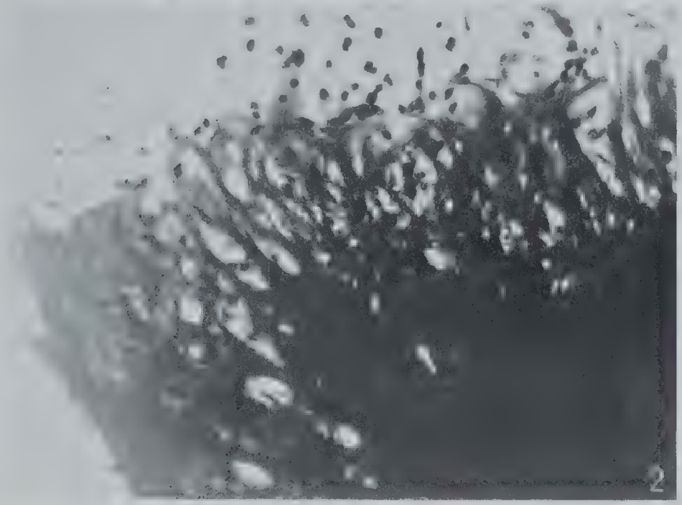
Fig. 14. Culture 889. 7-day culture from the heart of a 4-day embryo. Many muscle-cells near the explant are more or less disconnected and some are isolated. The mesenchyme extends out over 2 mm. Many of the muscle-cells were beating rhythmically at different rates. Janus green. Mitochondrial granules, rods, and threads. No fibrillæ were seen in the living cells. After fixation with iodine vapor some of the muscle-cells that had been beating contained cross-striated fibrillæ, some only smooth ones, and some none at all. ×117.

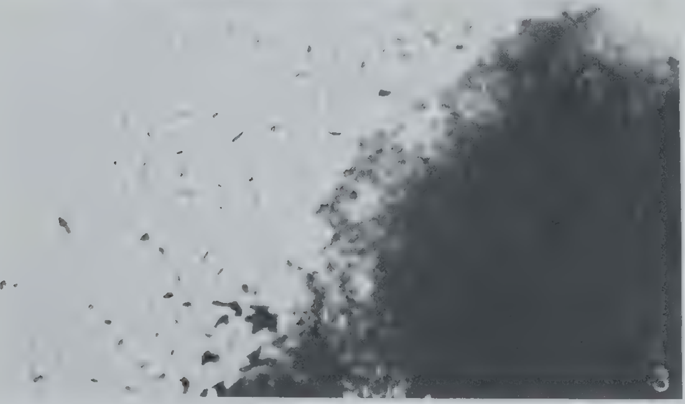
Fig. 15. Culture 889. Muscle-cells from region x in figure 14. Most of these cells were beating, each at its own rate. ×384.

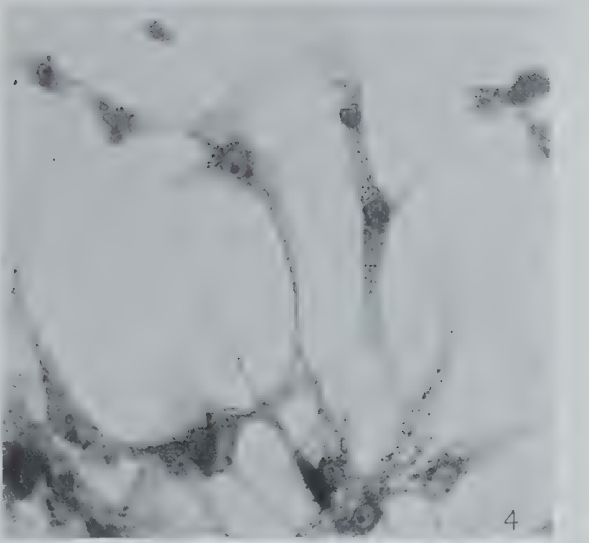
Fig. 16. Culture 889. Muscle-cells 1 and 2 from figure 15. These two cells were beating regularly, each at a different rate. A few smooth fibrillæ; mitochondrial granules. ×1160.

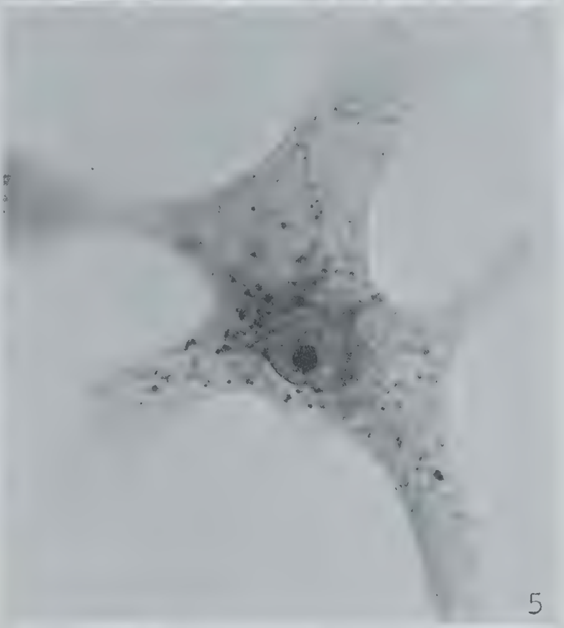
Fig. 17. Culture 889. Muscle-cell 3 from figure 15. This cell was beating. No fibrillæ were present. Mitochondria mostly granules. Few vacuoles. ×1160.











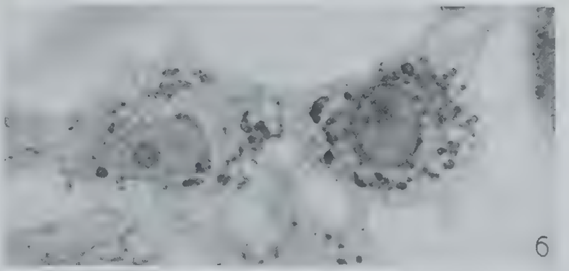


FIG. 1. Culture 827. 5-day culture from the heart of a 4-day embryo. The coarse muscle reticulum does not extend out as far from the explant as does the loose mesenchyme. Many cells were pulsating, some at rates different from that of the explant. Iodine fixation. Many cells show patches of glycogen. ×117.

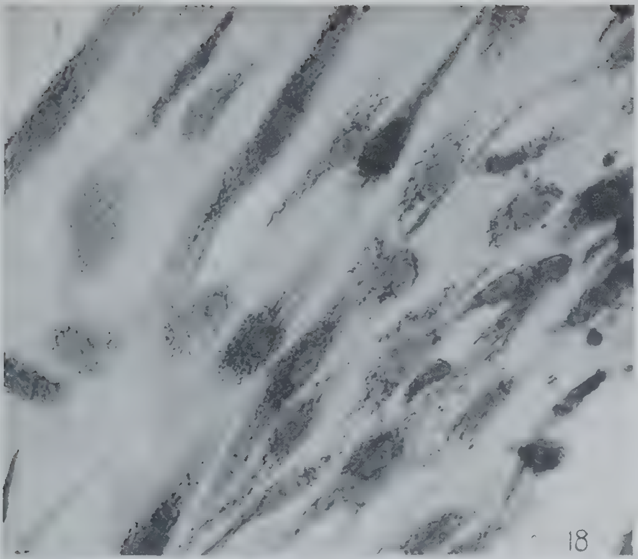
FIG. 2. Culture 875. 9-day culture from heart of a 7-day embryo. Coarse muscle reticulum. Mesenchyme cells degenerate and contracted into spherical forms. The muscle outgrowth contracted rhythmically with the explant. The contractions continued for over an hour at room temperature. Janus green, iodine. Very rich in glycogen. ×117.

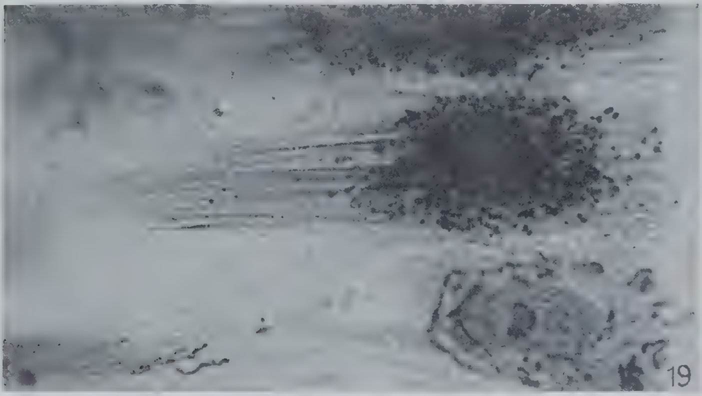
FIG. 3. Culture 827. Narrow border of muscle-cell membrane from same explant as figure 1, showing quite different type of muscle outgrowth. No glycogen. ×117.

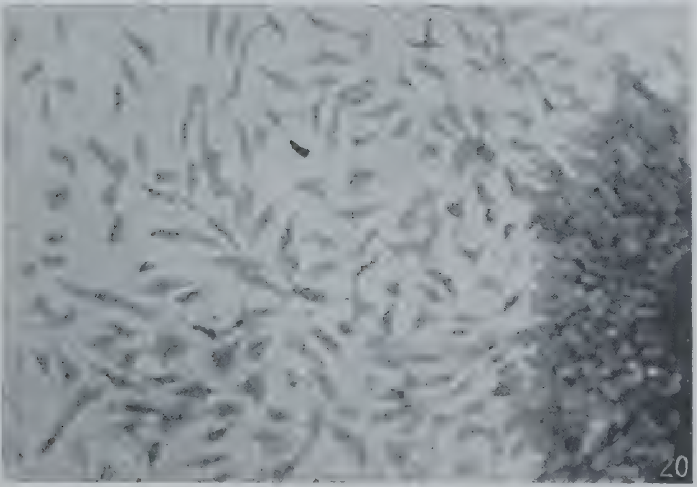
FIG. 4. Culture 875. Muscle-cells at periphery are more or less flattened against the cover-glass and show mitochondrial granules, rods, and a few threads. No fibrillæ or cross-striations. ×384.

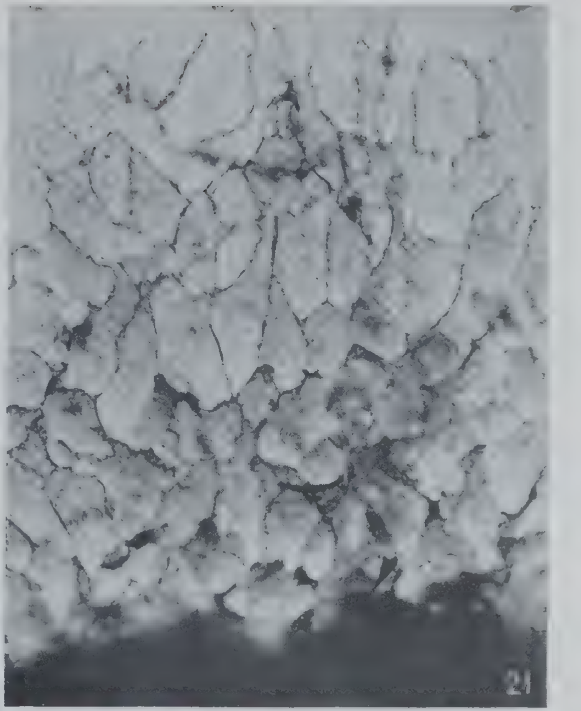
FIG. 5. Culture 875. Partially isolated muscle-cell from figure 4. Mitochondrial granules, rods and a few threads. No myofibrillæ. ×1160.

FIG. 6. Culture 875. Two cells from same culture, probably muscle-cells. ×1160.









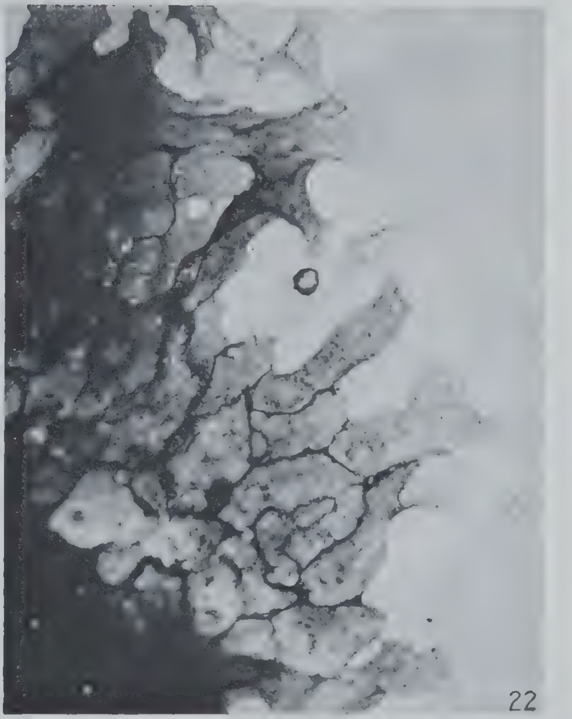


FIG. 18. Culture 889. Mixture of muscle-cells (*mu*) and mesenchyme (*me*). Some muscle-cells isolated and difficult to distinguish from mesenchyme. ×384.

FIG. 19. Culture 889. Muscle-cell (*a*) from figure 18, showing cross-striated and smooth fibrillæ. Mitochondria mostly granules and rods. ×1160.

FIG. 20. Culture 863. 7-day culture from heart of a 6-day embryo; *m*, heart-muscle membrane; *me*, mesenchyme. Silver nitrate. ×117.

FIG. 21. Culture 863. Edge of heart-muscle membrane with silver markings between cells. ×480.

FIG. 22. Culture 864. 8-day culture from 6-day heart. Silver nitrate. Before treating with the silver nitrate the whole membrane was beating rhythmically, and at the periphery some cells were beating at different rates, 110 and 140 per minute. Silver markings are seen between the cells. ×384.

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CONTRIBUTIONS TO EMBRYOLOGY, NO. 91.

CORRELATION OF EXTERNAL GENITALIA AND SEX-GLANDS IN THE HUMAN EMBRYO.

By Karl M. Wilson,
University of Rochester, School of Medicine and Dentistry.

With two plates.

CORRELATION OF EXTERNAL GENITALIA AND SEX-GLANDS IN THE HUMAN EMBRYO.

Coincident with the various studies that have been made on the development of the external genitalia in the human embryo, attempts have been made to lay down anatomical and developmental criteria by which the sex of the embryo might be determined from inspection of the external genitalia. While this is, of course, not a difficult point to decide after the external genitalia have assumed a definitive form, in the earlier stages of development, i. e. up to 14 or 15 mm. length, and often even later, all observers have found it extremely difficult and, in many cases, quite impossible. This is due to the fact that the features characteristic of one or the other sex develop rather slowly from the primitive external genitalia of the early embryo and, furthermore, considerable variation in time is encountered in the development of these characteristic structures when a number of embryos of apparently the same age are studied. Again, differentiation and development of the external genitalia not infrequently lag behind the general development of the embryo, and even behind the development of the sex-glands themselves. Consequently, it is not unusual to encounter specimens in which the sex can be easily determined from a study of the sex-gland, in the sectioned embryo, but in which such a diagnosis may prove to be quite impossible from inspection of the external genitalia alone.

Various suggestions have been offered from time to time for the differentiation of sex of young embryos by means of inspection of the external genitalia by observers who have studied this problem. A number of these suggestions have proved to be of distinct value, but they all fail in a number of cases to give the desired information. Thus, Nagel (1889) believed that the length of the urethral groove offered a clue as to the sex of a given embryo, this, according to him, being shorter in the male than in the female. He also drew attention to certain differences in the course of development of the labio-scrotal swellings in the two sexes. Herzog (1904) believed it possible to determine the sex of comparatively young embryos from a study of the direction of the phallus, and pointed out that in female embryos this structure tends to show a caudal curving, while in male embryos its long axis remains more nearly at a right angle to the long axis of the body.

Felix (1912) pointed out that the sex could be differentiated from the position of the ostium urogenitale and its relation, on the one hand, to the coronary sulcus and, on the other, to the anal opening. According to him, male embryos are distinguished by the fact that the distal circumference of the ostium urogenitale always lies in the coronary sulcus, while the proximal circumference becomes more and more distant from the anal opening. Female embryos, on the other hand, are recognized by the fact that the urogenital opening tends to retreat from the coronary sulcus by the gradual closure of its distal part, while its proximal part always lies close to the anal opening. Felix also pointed out that sexual differentiation of the external genitalia takes place quite gradually, and even in fairly advanced stages difficulties in diagnosis present themselves.

Among the disadvantages connected with some of the earlier work on this subject might be mentioned the fact that a number of the observers based their conclusions on the study of comparatively small series of embryos. In 1921 Spaulding studied a large series of embryos in the collection of the Carnegie Laboratory of Embryology with particular reference to the development of the external genitalia and the differentiation of the sexes. This material comprised embryos of all sizes, from 14 mm. to 100 mm. in length (crown-rump), but the greatest number were from 14 mm. to 50 mm. in length, comparatively few of the older stages being included.

From his study of this material he found he could separate the specimens into two groups, stage for stage, on the basis of the external genitalia, particularly with reference to the length of the urethral groove, back to a period as early as that represented by the beginning of the genital tubercle. He concluded that those with the longer urethral groove were males and those with a relatively shorter groove were females. According to him, "there is a morphological sex difference in the external genitalia of the human embryo practically from their first appearance, characterized by marked difference in the length of the urethral groove upon the caudal slope of the genital tubercle. In some embryos the distal end of the groove extends into the glans region (males), while in others it does not reach to this region (females)." He also drew the conclusion that, "from the first appearance of the genital tubercle, there apparently is no *indifferent* or *unindifferentiated* period in the development of the external genitalia before they acquire their definite male or female characteristics."

Unfortunately, in many of the cases in which Spaulding made a diagnosis of sex from a study of the external genitalia, he was not able to confirm this by a microscopic study of the sex-glands. A certain percentage of his material was unsuitable for histological preparation, while other embryos had not been sectioned at the time his study was completed. However, the histological preparations available at the time appeared to confirm satisfactorily the diagnosis made from inspection of the external genitalia.[1]

On account of the apparent reliability of the criteria laid down by Spaulding as a means of differentiating the sex of young human embryos, they were adopted as a routine procedure in the examination of all specimens brought to the Carnegie Laboratory, and have also been employed to some extent in other laboratories. As the result of further study of this problem, we are now in a position to discuss more fully the value of Spaulding's suggestions on the differentiation of sex.

In studying this question a series of 109 embryos in the Carnegie collection has been examined, these embryos being of various sizes and ages. Only those specimens in which there had been a careful examination of the external genitalia before the embryo was cut into serial sections have been made use of. A diagnosis of the probable sex of the embryo had been made from that examination, and this diagnosis has been either confirmed or refuted by the subsequent histological study of the sectioned specimens. It is the purpose of this communication to present the results of these findings and to determine the reliability of a diagnosis of sex that is made from an inspection of the external genitalia alone.

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[1] Only since my paper went to press has the work of Alfred Szenes (Morphol. Jahrb., Bd. 54, Heft 1, 65–135) appeared in this country. This was based on the study of a series of 57 male embryos ranging in size from 10.4 to 91.2 mm. (crown-rump length), and 59 female embryos, ranging from 7.3 to 200 mm. (crown-rump), with reference to the development of the sexual organs and particularly the external genitalia. Szenes lays down a number of criteria for the determination of the sex of a given specimen and emphasizes the changing relations of the genital folds and the genital (labio-scrotal) swellings during the course of their development as a particularly valuable diagnostic guide. He believes these signs to be reliable in all embryos from 18 mm. upward. His findings differ from those of Spaulding in a number of important respects.

The diagnosis had been made by various members of the laboratory staff in the course of the routine examination of the material brought in for study. In a number of instances it was made by Spaulding in the course of his work on this subject. In most instances I have been able to check these facts from photographs of the external genitalia; in a few cases it has not been possible to do this and I have been obliged to rely on the note made by the one who examined the fresh embryo in the first place.

Included in our series were embryos of relatively large size, 52 mm. to 113 mm. C. R. length. In this group 15 specimens were studied. In the case of these larger embryos no difficulty is ordinarily encountered in making a diagnosis of the sex by even a casual inspection of the external genitalia, so it was not thought necessary to study a larger series. In each specimen in this group the microscopic findings readily confirmed the diagnosis previously made from external examination.

In presenting the results obtained from the examination of the smaller embryos in our series, they will be discussed in length groups of 10 mm. In the length group of 41 mm. to 50 mm. there were 10 specimens. In general, the diagnosis made from inspection of the external genitalia was confirmed by microscopic examination of the developing sex-gland, but at the same time an important discrepancy was noted. In one embryo in this series, 50 mm. C. R. length, the external genitalia were of the typical female type, according to the usually accepted criteria. Microscopic examination however, in spite of a certain degree of maceration, revealed the fact that the sex-gland was beyond doubt of the male type (figs. 1 and 2). From the study of such a small series as we have in this group (10 specimens) it would be unfair to say that the possibility of error in the diagnosis made from external examination is as great as 10 per cent. However, even this single discrepancy indicates very definitely that this possibility of error does exist, even in embryos of a well-advanced state of development.

The next group length, including embryos from 31 mm. up to 41 mm., comprised 25 specimens. In this group an increasing number of discrepancies between the diagnosis made from external examination and that made from microscopic examination was noted. Thus in 9 instances it was found that the histological picture was at variance with the supposed sex as diagnosed from the appearance of the external genitalia, an error of 36 per cent. Each of the 9 embryos had been diagnosed as female from inspection of the external genitalia, and in each case the sex proved to be male. Thus the error was always in the same direction. Such a high percentage of error of course indicates the impossibility of making an accurate diagnosis of the sex of embryos of these sizes from external examination alone.

The findings would also seem to indicate that the final differentiation of the external genitalia of the male may be delayed to a period considerably later than that in which differentiation has occurred in the sex-glands, and that development of the external genitalia in many instances does not keep pace with the development of the internal genitalia.

In the next length group, from 21 mm. to 30 mm. C. R. length, 35 specimens were studied. Again, a relatively high percentage of discrepancies between the original diagnosis, as made from the external genitalia, and the actual sex was discovered, 10 such specimens being encountered, or an error of 28.5 per cent. In one additional specimen the diagnosis was still in doubt after examination of both the external genitalia and the sex-glands. In this group the error was in general in the same direction as in the preceding group, that is, supposed females proved to be males. In two instances, however, the reverse proved to be the case, supposed males proved to be females.

In the series Spaulding studied he found a greater percentage of females than males in the embryos of from 14 mm. to 50 mm. This can be readily explained on the basis that a considerable proportion of the supposed females were actually males.

The fourth group consisted of 24 embryos, from 16 mm. to 20 mm. in length. Here, also, discrepancies in diagnosis were found, though these were not so numerous as in the preceding groups, only 3 such instances being observed. In 2 the supposed female proved definitely to be male, while in the other the reverse was true. It must of course be recalled that in this group it is sometimes impossible to make a positive diagnosis from the microscopic examination, on account of the delayed differentiation of the distinctive sexual characteristics of the internal genitalia.

We have also studied a number of embryos, 14 mm. to 15 mm. in length, and have encountered discrepancies. In this group, however, many specimens would necessarily have to be put down as of doubtful sex, even after microscopic examination, so they will not be considered further.

The possibility of inaccuracies in the diagnosis of the sex can perhaps be made more evident from the study of a few typical examples of varying sizes. Thus, figure 1 represents the external genitalia of the embryo of 50 mm. C. R. length, mentioned above. Inspection of the structures depicted reveals the fact that the urethral groove does not extend into the coronary sulcus. This fact, according to Spaulding, should cause one to classify the embryo as a female. Furthermore, the proximal portion of the urogenital opening lies in intimate relationship with the anal opening, while the distal portion is retreating from the coronary sulcus. According to Felix, these facts together should cause one to classify this specimen as a female. However, figure 2, which represents a portion of a sagittal section of the sex-gland (in spite of a certain degree of maceration), reveals the features characteristic of the early testis, branching and interlacing sex-cords, a well-defined albuginea layer and a flattened surface epithelium showing no further signs of proliferation.

Figure 3 shows the external genitalia of an embryo which has also attained a considerable degree of development, being 39 mm. C. R. length. Judged by the criteria of Spaulding and Felix, mentioned above, this specimen should also be classified as a female. In addition, a distinct caudal decurvation of the phallus is noted (fig. 3A). This, according to Herzog, should be confirmatory evidence in favor of a diagnosis of female sex. A glance at figure 4, which represents a section through a portion of the sex-gland, again reveals, however, the definitely male characteristics of a young testis.

Figure 5 shows very distinctly the criteria ordinarily regarded as positive for the female in another well-developed embryo of 35 mm. length, and again the microscopic structure of the sex-gland is clearly of the male type (figure 6).

Figures 7 and 9 represent the external genitalia of somewhat younger embryos, 29 mm. and 23.7 mm. C. R. length, respectively. In figure 9 a well-defined coronary sulcus is not noted, although its region is indicated. In neither of these specimens does the urogenital opening extend to the tip of the phallus; hence, according to Spaulding, they should be classed as female; according to Felix, as indifferent. Figures 8 and 10 show very clearly, however, that the sex-glands are of the male type.

SUMMARY.

While it is true that in this rather large series of specimens the diagnosis of sex, made from an examination of the external genitalia, has been confirmed in a fairly high percentage of the specimens by an examination of the sex-glands, on the other hand a high percentage of error has also been noted, the errors in diagnosis being particularly numerous in embryos from 21 mm. to 40 mm. in length.

In our series of embryos in this length group (21 mm. to 40 mm.) 60 specimens were examined. The sex was correctly diagnosed by external examination in 41 of these specimens, but the diagnosis was erroneous in 19 specimens, or 31.6 per cent. Such a high percentage of error at once reveals how unreliable and inaccurate are the criteria at present depended upon in diagnosing the sex of a given embryo by external examination, even those in a well-advanced state of development. Indeed, our experience leads us to feel that it is quite impossible to do this with absolute accuracy in all cases until the embryo has attained a crown-rump length in excess of 50 mm. In general, we have found that in cases in which the diagnosis of sex, as made from examination of the external genitalia, was at variance with the actual sex, as revealed by histological study of the sex-glands, the error has been in diagnosing males as females. This fact would apparently indicate that in many instances the development and differentiation of the external genitalia are markedly retarded as compared with the development of the sex-glands, and particularly so in the male embryo.

In only three instances did we find that the error in diagnosis occurred in the reverse direction, that is, that supposed males proved actually to be females. In each instance in which this was found to be the case, the diagnosis had been made by the one who examined the specimen when it was first brought to the laboratory and, as no satisfactory photograph had been retained, it was impossible for us to confirm or deny the original diagnosis.

While recognizing the frequency of retardation of development of the external genitalia in male embryos, one or two other possible factors that might apply to the embryos erroneously diagnosed as females should be mentioned. The possibility of external hermaphroditism can not be ignored. However, it would seem unlikely that such a possibility could apply to as high a percentage of embryos as we found in this series. Furthermore, in each specimen of our entire series both sex-glands were examined and in all cases were found to be developing along normal lines.

Another possibility to consider is that these supposedly female embryos actually represented specimens with defective external genitalia which would perhaps have exhibited such a defect as hypospadias had they continued to mature development. Again, however, the frequency would seem to be too high, and is certainly much higher than the incidence of such a defect in postnatal life. For these reasons, I am inclined to emphasize the retardation of development of the external genitalia in male embryos, and feel that this phenomenon occurs much more frequently than has hitherto been realized. As is well known embryologically, practically any structure in the developing embryo may, for as yet unknown reasons, be retarded in its growth and development and lag behind the development of other structures, which are proceeding at a normal rate of growth.

CONCLUSIONS.

1. The various criteria laid down for determining the sex of a given embryo from examination of the external genitalia are extremely unreliable and can only be applied with accuracy to embryos exceeding 50 mm. crown-rump length.
2. When, following the criteria of Spaulding, a diagnosis of male sex is made in a given case, the percentage of error is much lower than when an embryo is diagnosed according to his criteria as a female.
3. Retardation of the development of the external genitalia in male embryos is a very frequent phenomenon.

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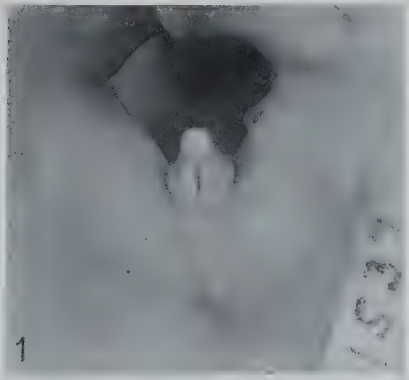

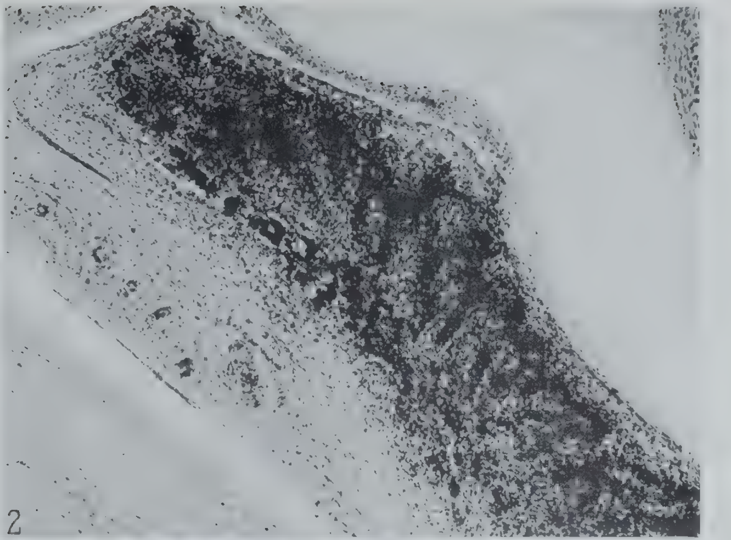

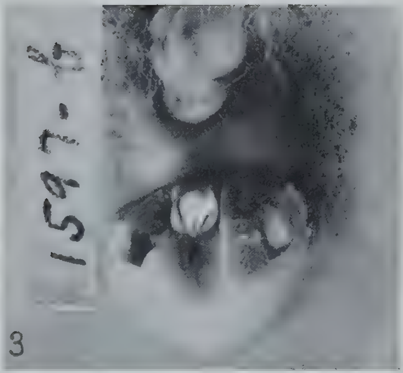

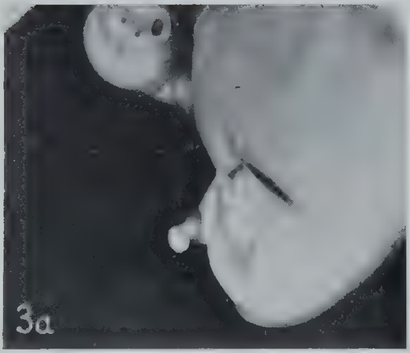

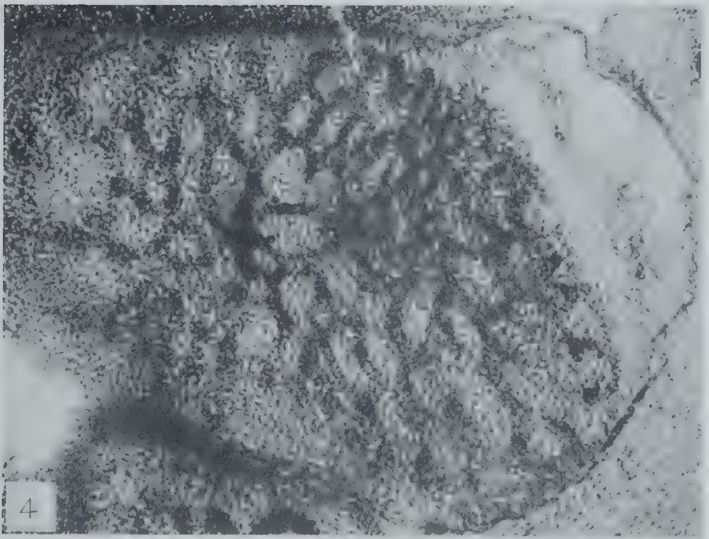

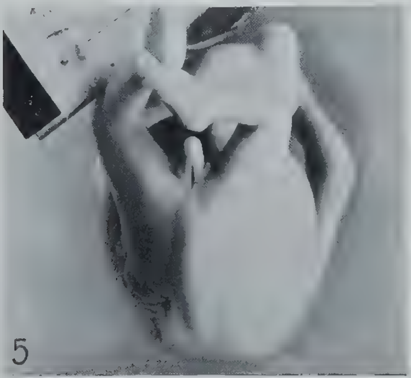

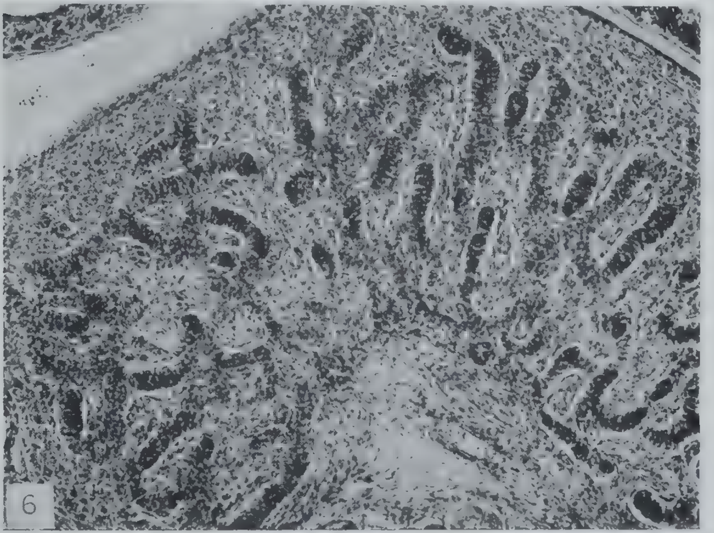

1. Embryo No. 1537 (50 mm.). External genitalia.
2. Same. Sex-gland (sl. 43). ×100.
3. Embryo No. 1597b (39 mm.). External genitalia.
3a. Same, lateral view. Note caudal decurvation of phallus.
4. Same (sl. 30, sec. 1). Sex-gland. ×100.
5. Embryo No. 210 (35 mm.). External genitalia.
6. Same (sl. 87, row 1, sec. 2). Sex-gland. ×100.

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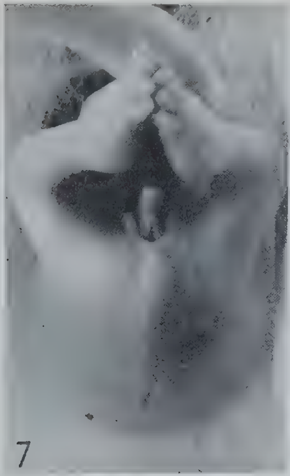
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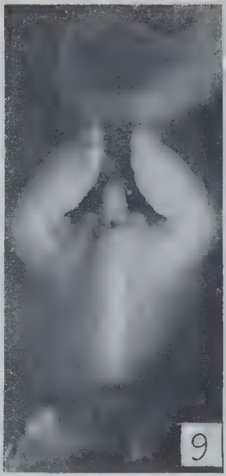
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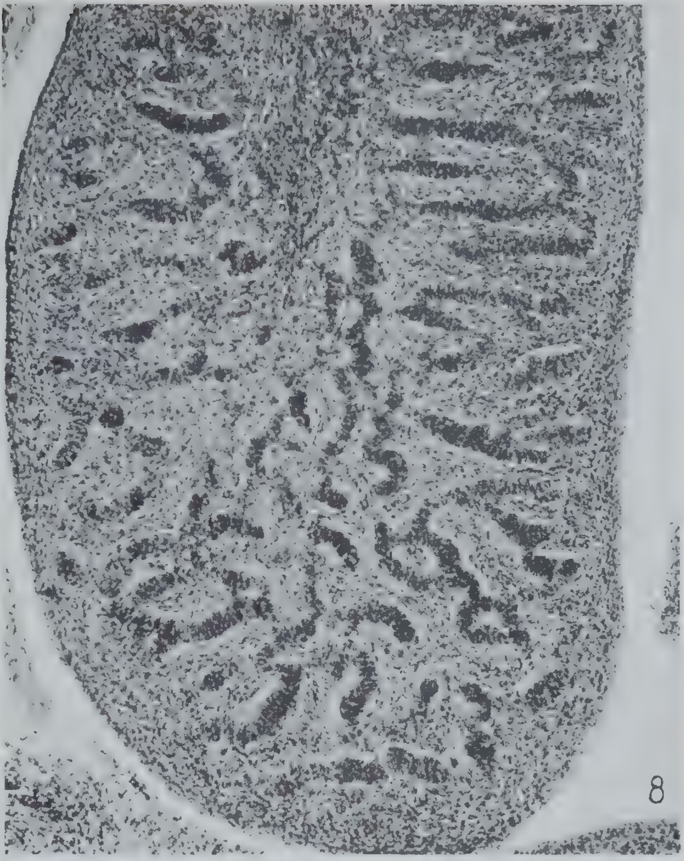
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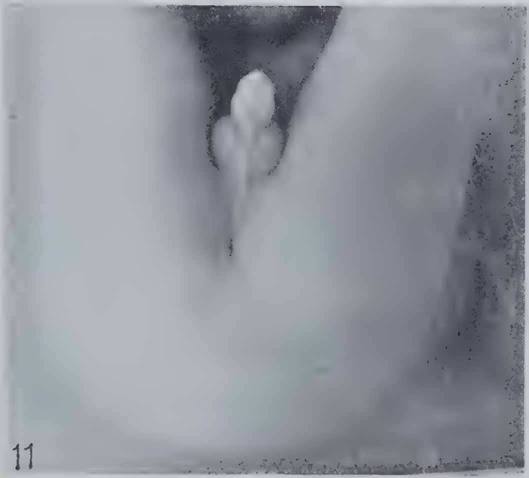
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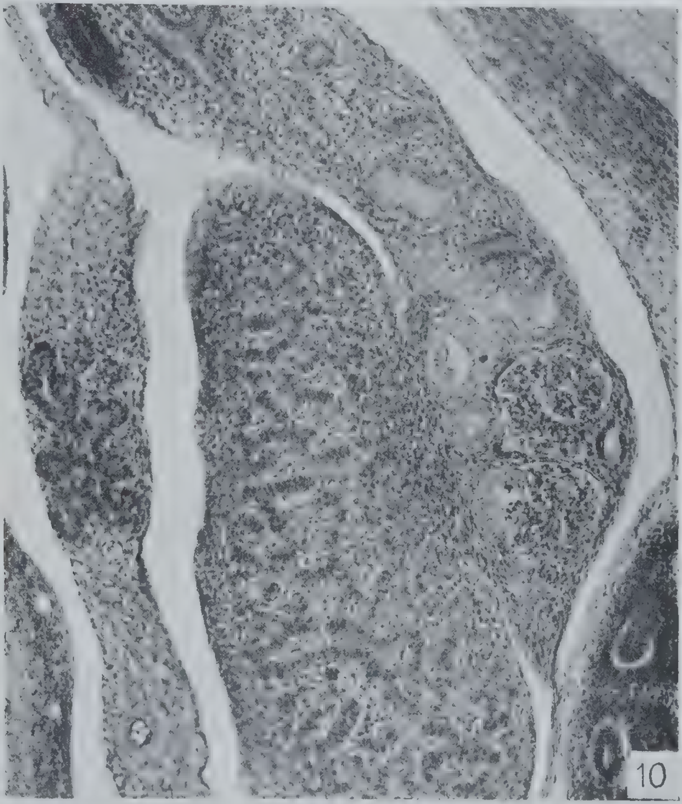
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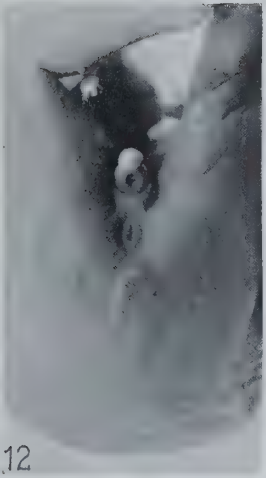
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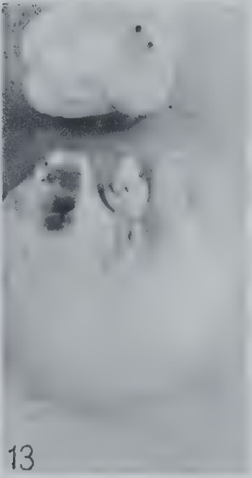
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7. Embryo No. 950 (29 mm.). External genitalia.
8. Same (sl. 48, row 2, sec. 3). Sex-gland. ×100.
9. Embryo No. 2393 (23.7 mm.). External genitalia.
10. Same (sl. 26, row 1, sec. 1). Sex-gland. ×125.
11. Embryo No. 1183 (56 mm.). External genitalia, male.
12. Embryo No. 538 (50 mm.). External genitalia, male.
13. Embryo No. 1686 (44 mm.). External genitalia, male.

(Figures 11, 12, and 13 are typical male embryos of the indicated sizes and are introduced simply for comparison. In each instance the diagnosis was confirmed by the microscopic appearance of the sex-glands.)

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CONTRIBUTIONS TO EMBRYOLOGY, NO. 92.

THE "MILLER" OVUM—THE YOUNGEST NORMAL HUMAN EMBRYO THUS FAR KNOWN.

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Baltimore, Maryland.

With eight plates and three text-figures.

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THE "MILLER" OVUM—THE YOUNGEST NORMAL HUMAN EMBRYO THUS FAR KNOWN.

INTRODUCTION.

In 1909 a patient complaining of dysmenorrhea was curetted in the Woman's Clinic at the University of Heidelberg, and, subsequently, in a microscopic examination of the scrapings, Dr. J. W. Miller, at that time assistant in the clinic, discovered the presence of a very young ovum. A brief description of the specimen, with a carefully made drawing of one of the sections, was published by him as part of a study dealing with the function of the corpus luteum (Miller, 1913). In that paper the importance of the specimen was pointed out and its principal embryological features were clearly and correctly noted. Since, however, this specimen provides us with a picture of the implantation site before the decidual reaction of the endometrium has taken place, and since it is evidently a normal ovum, the youngest thus far known, there is clearly a need for a fuller description and particularly of more complete illustration of the material. It was therefore with the greatest pleasure that I took advantage of Dr. Miller's permission to study the original sections, and at his suggestion I have prepared this account, together with the photographic reproductions, so that a record of the detailed structure of this unique specimen may be readily available to the embryologist.

CLINICAL DATA AND AGE OF SPECIMEN.

The clinical history of the case, as given by Dr. Miller, is as follows:

Frau S. M., 26 years old. Two spontaneous deliveries, the last in September, 1907. Entered hospital January 8, 1909.

Menstrual History: Periods began at 15, at first lasting 2 days, scanty; at present 5 days, profuse, accompanied by abdominal pain and backache. Intermenstrual discharge. Menstrual type, usually about 3 weeks. Last period, "vor Weinachten," 1908.

Anamnesis: After last delivery nursed her baby 9 months; there then occurred a 1-day period (preceding Whitsuntide, 1908), then 9 weeks amenorrhea, followed by bleeding lasting 14 days. Thereafter, every 14 days menstruation, lasting 5 to 6 days, accompanied by severe pain. In October moderately profuse bleeding, lasting 3 to 4 weeks. At present profuse menstruation occurs every 24 days, lasting 5 to 6 days. Patient complained of above mentioned dysmenorrhea and of persistent backache and general weakness.

Operation: Curettage and Alexander Adams suspension of uterus on January 14, 1909, with subsequent history of regular menstruation and no further dysmenorrhea.

From the above clinical data one can say with certainty that the specimen is more than 6 days old—the duration of the patient's residence in the hospital prior to operation. Further than this there is no critical evidence upon which to determine the age of the specimen. However, although it may be hazardous to form an estimate of age from the endometrial picture, it may be stated that the endometrium is of the late premenstrual type and not more than 2 or 3 days before onset, as will be described later; and that, since the patient's cycles had recently been of 24 days' duration, her last period probably began at least 21 or 22 days (menstrual age of ovum) before the operation—that is, December 23 or 24. This would harmonize with the patient's statement that it began "vor Weinachten." In the absence of pregnancy, the next period should have begun 24 days later, on January 16 or 17. Therefore, if one assume the occurrence of ovulation to be midway between periods, in this case 12 days before the expected period (i. e., January 4 or 5), it would follow that the ovulation age of this ovum is 10 or 11 days. In forming this estimate, it must be remembered that we are depending not only on an endometrial picture, which may have been modified by the existence of pregnancy, but also on the very uncertain time of ovulation; and finally the menstrual cycle itself had been quite irregular and one can not be sure that the 24-day periods of the last two months would have been repeated.

THE MATERIAL.

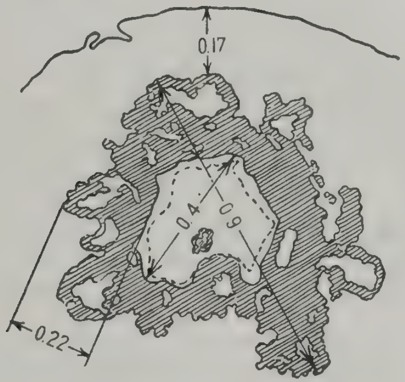
The tissue in which Dr. Miller made his important discovery consists of a group of fragments of curetted endometrium, which were embedded together in a block of paraffin and cut in group sections 10μ thick. Each of the five slides of this material that were rescued consists of three larger fragments of endometrium, about 15 by 4 mm., together with several smaller fragments. The fragments were not oriented before cutting, and so in the sections they are found cut in various planes; neither were they saved in series. By superimposing them, however, one can unquestionably make out their proper sequence, chiefly by matching selected glands. When the slides came to me from Dr. Miller they had already been numbered 4 to 8, and I concur entirely in the sequence thus designated by him. Throughout this paper they will be referred to by those numbers.

The ovum is situated in the tip of one of the larger fragments. If this fragment had been 2 mm. shorter the ovum would have been completely missed. A photograph of this tip, as it appears on each of the 5 slides, is shown on plate 1, figures 4 to 8, arranged in their proper order, the first being the most superficial with reference to the summit of the implantation site. This is rendered certain by their respective size and by the sloping surface of the endometrium. It is thus clear that the sections of the tip of this fragment were cut tangential to the implantation site, passing transverse to the mouths of the uterine glands. The one disadvantage of this plane of section is that it does not show the point of entrance of the ovum and gives no information as to the closing cap ("Gewebspilz" or "Schluss-coagulum") of Peters. By good fortune, 4 of the sections, coming very near together, pass through the inner cell mass and the region of the body-stalk; the part of the ovum that is not represented is the more superficial part, which would have shown only the trophoblastic shell and which is sufficiently well portrayed in the recovered sections. Three of the sections were injured mechanically, uncomfortably close to the region of the ovum, apparently during the process of staining and mounting. The tissue otherwise is in a perfect state of preservation and presents a beautiful stain reaction to hæmatoxylin and eosin.

MEASUREMENTS.

The measurements of the ovum, given by Miller in his original publication, were made by Professor Keibel,[1] at that time in Freiburg. I have checked these and have obtained results that are substantially the same; the only deviations being ones that could be accounted for by differences in the selection of points of measurement. My own measurements are given graphically in the accompanying text-figures 1 and 2.

The greatest external diameter of the ovum, measured from tip to tip of the most outlying plasmoditrophoblast, is 0.9 mm. If one disregards the prolonged syncytial loops, the average external diameter is about 0.6 mm. Moreover, it seems quite probable that the syncytial trophoblastic processes, as seen in the Miller ovum, are an outgrowth subsequent to its penetration into the endometrium. If this is so, then the size of the ovum at the time of penetration was not greater than its present cytotrophoblast, the extreme dimensions of which vary from 0.37 to 0.45 mm. Under conditions of fixation and imbedding in paraffin such as exist in the Miller ovum, the size of the egg when it left the ovary can be estimated by measuring mature eggs in sections of the ovary. The largest one that we have at present in this laboratory has a diameter of 0.12 mm. It is usually stated that they range from 0.15 to 0.25 mm. If we take the smallest size of the ripe ovarian egg (0.12 mm.), then its subsequent increase in size while free in the tube and uterus is not greater than 5 times its original diameter, and probably a threefold or fourfold increase would be more nearly correct.



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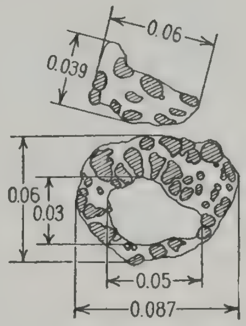

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FIG. 1.—Dimensions of the Miller ovum given in millimeters, as measured on slide 3. × 50.

FIG. 2.—Dimensions of the amniotic vesicle (below) and the supposed yolk-sac (above) in millimeters, as measured on slide 4. × 250.

For the greatest diameter of the interior of the trophoblastic shell—the chorionic cavity—I obtain 0.4 mm., about the same as that given by Keibel. An average diameter of the trophoblastic lumen, however, is 0.35 mm., and an average thickness of the trophoblastic shell, including both the cytotrophoblast and the plasmoditrophoblast, may be given as 0.22 mm. The outer margin of the plasmoditrophoblast lies 0.17 mm. below the uterine surface. It is of interest to note how very near the surface the ovum lies at this time. This measurement would doubtless be even less if we had a section passing transverse to the summit of the implantation site. In reality, the ovum lies in the compacta, only just beneath the surface epithelium, and it is not until later, following decidual reaction, that one meets with a thicker decidual capsule.

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[1] In Miller's original article it was stated that the measurements were made by Professor "Hechel." This was a misprint, it should have been *Keibel*.

The amniotic vesicle is best shown on slide 4 (fig. 2) and its outer dimensions are 0.087 mm. wide and 0.06 mm. high. Keibel's measurements were 0.095 by 0.072 mm. The amniotic cavity is 0.05 mm. wide and 0.03 mm. high. The triangular mass, which is thought to represent the yolk-sac and which doubtless does not represent its full size, has the dimensions of 0.06 by 0.039 mm.

The drawing published by Miller in his original article is stated to be an enlargement of about 185 diameters. This evidently was a typographical error, or perhaps account was not taken of reduction in the process of reproduction. As it was finally printed, it represents an enlargement of approximately 86 diameters. At the time Dr. Miller's paper was published (1913) he was in Brazil and this prevented his seeing the proof. There thus resulted uncorrected typographical errors. He has sent me a list of these; in addition to those I have just mentioned, there are the following in the 8 paragraphs of smaller type describing this specimen: Middle of first paragraph should read: "so die Einbruchsstelle . . . *nicht* zur Darstellung gerlangt ist;" in the next sentence one should read "in *vier* Präparaten"; in the seventh paragraph, "auf den fünf Schnitten" should be transposed to the line above, following "findet"; in the fifth line of the same paragraph "parallel" should read "schräg"; in the figure legend, "solider Embryonalknoten" should read "Embryonalanlage."

TROPHOBLAST.

If, in man, the egg passes through the fallopian tube in about 3 days, as Corner (1921) has shown to be the case in the pig, it must reach the uterus by the fourth day. The 10- or 11-day ovum with which we are dealing must therefore have been a resident in the uterus 7 or 8 days. Just how large a part of this time it was lying free in the uterine cavity, bathed by the secretion products of the uterine glands, the "embryotrophe," we can not yet say. We may assume, however, that important changes occur in the epithelial wall of the blastocyst during this period, and that it is due to these changes that the egg is enabled to penetrate the surface epithelium of the uterus, either mechanically wedging itself between the epithelial cells or, by cytolytic action, eroding a sufficiently large area to permit its entrance into the underlying stroma. It is probable that the picture of the penetrating egg, given us by von Spee (1901) for the guinea-pig and more recently by Celestino da Costa (1922) for the hedgehog, approximately represents the phenomenon of penetration as it occurs in man. Judging by the small amount of disturbance that has thus far been produced in the stroma immediately surrounding the Miller ovum, we may assume that it only recently reached its present position and had spent the brief interval in the elaboration of the trophoblastic syncytium preliminary to a more vigorous attack upon the maternal tissues.

We already know from the pig ovum that the trophoblastic ectoderm is much more precocious in its differentiation than the embryonic ectoderm—the inner cell mass—and the reason for this obviously lies in the part that the trophoblast plays in the process of implantation, and nutrition. This fact is very striking in the Miller ovum, as can be seen in the low power views shown in figures 4 to 8. The trophoblast, as it appears under a higher power, is shown in figures 9 to 12, and in further detail in figures 13 to 15. It will be seen that the simple epithelial wall of the original blastocyst has, on the one hand, become lined with a mesoderm-like membrane and, on the other, has projected out against the surrounding stroma irregular multinuclear epithelial processes or syncytial loops. It is the original wall of the blastocyst that seems to be continued as the cytotrophoblast of Hubrecht and that later forms the Langhans layer of the placental epithelium. It is well shown as a single layer of uniform cuboidal cells in portions of the wall in figure 9 and also in figure 13, where the section passes transversely to its surface. By studying other portions of the wall one can find it cut in various planes. Everywhere from the surface of this simple layer of epithelium there arises a luxuriant growth of epithelial syncytium—the plasmoditrophoblast of Hubrecht, the implantation syncytium of Grosser. The transition between the two is very well shown in the oblique section of the wall in the upper right quadrant of figures 10 and 11.

The striking peculiarity of these multinucleated processes of plasmoditrophoblast is their tendency to wall off clear spaces, small near the wall of the ovum and larger as one approaches the periphery. These are the well-known lacunæ. It was suggested by Miller that these lacunæ represent capillary spaces, clothed and pressed outward by the syncytium following the obliteration of the capillary walls.

If, in figures 9 to 13, one examine carefully the primitive syncytium at its transitional base, where it is elaborated from the cytotrophoblast, one finds everywhere a great many small, fluid-containing spaces, which tend to separate the cell units off from one another. In addition to the small spaces, there are larger ones, and in fact one can pick out transitional sizes all the way to large lacunæ. This is particularly well shown in figure 12. The smaller spaces might be considered as vacuoles of intracellular origin. Those of intermediate and larger size, however, lack the necessary characteristic of degeneration vacuoles and fluid inclusions, in that one can not be sure that they are entirely surrounded by cytoplasm. One can not eliminate the possibility of their communicating with and being a part of the system of intercellular spaces. In figures 13 and 15, where larger lacunæ are shown, one can see how the irregularity of the lacunar walls produce the appearance of isolated intracellular vacuoles, which in reality are recesses from a common cavity, the effect of separate spaces being produced by the tangential section of the wall. Apparently these lacunæ present the histological picture of a process by which a solid mass of trophoblast becomes converted into a sponge-like syncytium, thereby offering a maximum surface of exposure of the constituent cytoplasm to the surrounding fluids. If one compare the lacunæ of the Miller ovum with the implantation cavity of older specimens (Kleinhans, Teacher-Bryce I, Peters, and Schlagenhaufer-Verocay), it is possible to follow their further enlargement and coalescence as they become flooded and distended with blood. Along with the distention of the lacunæ the syncytium becomes spun out into relatively thinner irregular strands, which, however, preserve a protoplasmic continuity between the trophoblastic wall of the ovum and the surrounding uterine stroma.

Another peculiarity of the plasmoditrophoblast is its capacity for eroding capillaries and establishing open communication with the maternal vessels. In the upper part of figure 13 the trophoblast can be observed approaching the capillary, and figure 14 shows clearly a capillary wall already eroded and communicating with the lacunæ of the trophoblast. It contains two leucocytes. As with other capillaries surrounding the implantation site, there are few or no red blood-cells present. Apparently, the lacunæ have not as yet been flooded with blood. Nothing can be said regarding the relations of the beginning implantation cavity with any particular vessels of the maternal blood system, such as has been pointed out by Teacher in nearly related stages. The capillaries in some other fragments of the endometrium remote from the implantation site happen to be fully distended with red cells. Where this is so, one can recognize long straight vessels lying between the uterine glands and running parallel to them. These vertical stems are united by branching arches at the level of the junction of the compacta and future spongiosa layers. Such arches should lie directly beneath the implantation site, but the plane of the sections and the incompleteness of the series makes it impossible to say whether this is so or not.

The plasmoditrophoblast behaves differently toward the uterine glands than toward the capillaries; it does not open into them or erode their surfaces. To the left of the middle of figure 13 one can see the syncytium abutting directly against a uterine gland and spreading around it without showing any injury to the uterine epithelium. The same thing is shown in figure 14, where contact between the syncytium and the uterine epithelium appears to cause no change in the latter.

A careful examination was made to see the effect of the syncytial loops upon the surrounding uterine stroma. I can not see any evidence of destruction or ingestion of the stroma cells. The syncytium apparently simply presses the stroma outward, as described by Miller. There is, however, in the vicinity of the ovum, an invasion of the stroma by a moderate amount of mononuclear leucocytes. Also, many of the stroma cells show an increase of cytoplasm characteristic of young decidual cells. Some of these can be seen in figure 15. This beginning of decidual reaction is found only in the neighborhood of the ovum. There is no sign of it yet in the fragments from other portions of the uterus.

If we now study from the cytotrophoblast inward, we find it lined with a well-organized primitive mesoderm, which is irregularly retracted from the surface, evidently due to shrinkage incurred in the dehydration and imbedding of the specimen. In addition to lining the chorionic cavity, the primitive mesoderm sends irregular trabeculæ across the lumen connecting the lining membrane with a similar membrane enveloping the inner cell mass. Thus one might speak of a parietal and a visceral layer of the primitive mesoderm that can be identified even as early as this. I know of no particular advantage in using such a designation, however, and it would possess the disadvantage of confusing these layers with the somatopleure and splanchnopleure of the cœlomic cavities, which are formed in a different manner and with which they are not strictly analogous.

It is this same primitive mesoderm which separates the inner cell mass from, or attaches it to, the chorionic wall. In other words, it forms the body-stalk, as can be seen in figures 11 and 12. Everywhere throughout the meshes of this primitive mesenchyme one finds a fine, granular, eosin-tinged coagulum, representing the albuminous fluid that fills these spaces. In figure 11, to the right and above the inner cell mass, one of the primitive mesenchyme cells is undergoing mitotic division and presents a beautiful picture of the anaphase stage. From a study of this specimen it is difficult to derive this highly differentiated primitive mesoderm, which appears to be characteristic for man, from the inner cell mass, long before there is any primitive streak or any true mesoderm. It must have either separated off from the inner cell mass during the formation of the segmentation cavity or have been derived from the trophoblast. Since it is, in reality, so largely concerned in the differentiation of the trophoblastic structures, the latter is the more probable explanation. Eventually it furnishes the connective tissue elements of the umbilical cord, the chorionic membrane, and the vascular stroma of the chorionic villi, never becoming an actual part of the body of the embryo. Later, in its meshes is formed the exocœlomic space, walled off by the mesothelial transformation of its trabeculæ, exactly in the same way that the perilymph spaces are formed in the ear and the arachnoid spaces around the central nervous system.

THE EMBRYO.

If one compare the Miller ovum with other mammalian forms of which the structure of the early blastocyst is fairly well known, it is apparent that the single-layered trophoblastic ectoderm which forms the wall of the early blastocyst has, in the Miller ovum, undergone elaborate modification to form cytotrophoblast, plasmoditrophoblast, and perhaps also the web of primitive mesoderm found within the cavity of the ovum. In contrast to the marked differentiation of the trophoblastic ectoderm, the embryonic ectoderm or inner cell mass has undergone very little change. It is this small inner cell mass that eventually forms the entire body of the embryo. Four of the five sections that were saved pass through it. The sections are 10μ thick; thus we have 40μ of the inner cell mass represented. Since the greatest width of the inner cell mass is 87μ, and assuming that this is equal to its greatest length, there are 5 sections unaccounted for. Though one regrets that the series is not complete, the sections that we do have indicate the essential anatomy of the mass.

In the schematic profile reconstruction shown in figure 3 the existing sections are plotted in their respective positions for the purpose of indicating their extent, topography, and sequence. The 4 sections passing through the embryo are shown in figures 9 to 12 and will be described in their order, passing from the more superficial to the deeper lying ones.

Section 1, shown in figure 9, passes through that part of the inner cell mass nearest the surface of the implantation site, and one can see a group of 11 ectodermal cells, cut tangential to the wall of the amniotic vesicle, with perhaps just the tip of the cavity showing. These cells are surrounded on all sides by a sheet of primitive mesoderm. To the left of the mass there is a crescentic shrinkage space. The border of this space, toward the inner cell mass, shows a row of cells which I interpret as belonging to the primitive mesoderm, although they seem more compact and more like ectoderm than does primitive mesoderm in other places. It is possible that this appearance is to be explained by the oblique direction in which they are cut. I point this out because there exists a difficulty in definitely identifying the presence of an endodermal mass in this ovum, which leads one to be alert to every possibility. It is likely that the compact, orderly, epithelial-like arrangement of this particular group of cells came about through the formation of the adjacent shrinkage space. These cells are certainly entirely separate from the inner cell mass, which would not be the case if they represented endoderm. It will be noticed that section 1 lies superficial to the region of the body-stalk and that the inner cell mass lies free toward the center of the ovum, with no definite attachment to the cytotrophoblast.

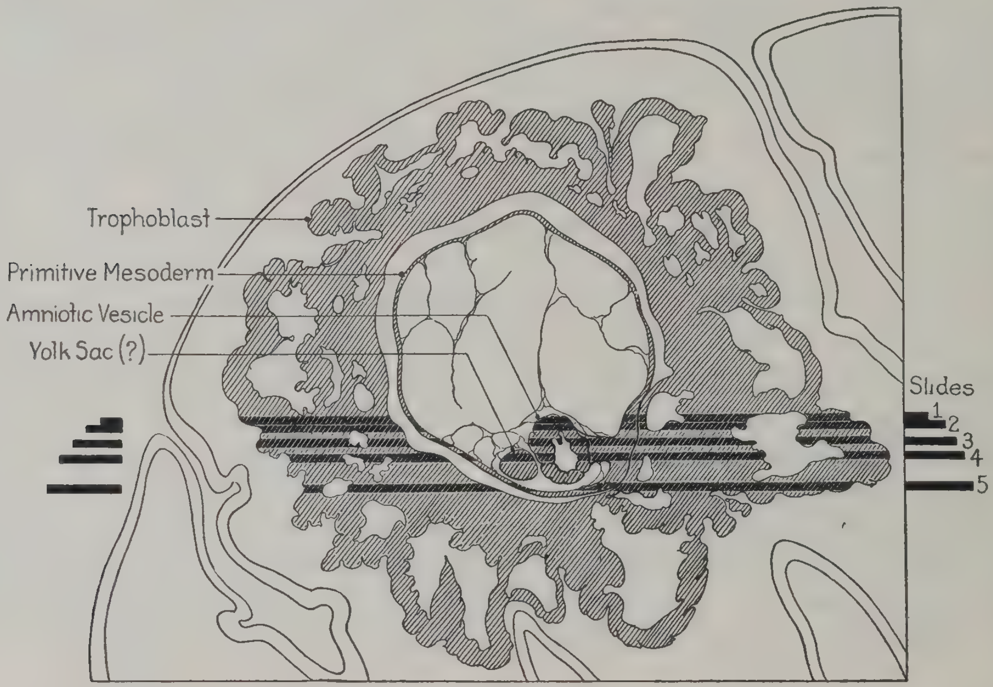

Fig. 3.—Schematic profile reconstruction of the Miller ovum, showing level of the individual sections and proportion of the entire mass they represent. The trophoblast is drawn in on the assumption that it is the same in amount and arrangement in the schematic plane represented as in the actual plane of sections. This schematic plane is approximately parallel with the opening ducts of the uterine glands. It should show the point of entry through the surface of the endometrium. No attempt was made to represent this, as there was no data from which to reconstruct it.

The section shown in figure 10 apparently is the next section deeper in the ovum. Here one can definitely make out a mass of ectoderm forming the wall of the inner cell mass—in other words, the amniotic vesicle—and the tip of the amniotic cavity can be recognized. As in the preceding section, the inner cell mass is completely ensheathed by primitive mesoderm, and below can be seen part of the body-stalk, consisting of primitive mesoderm cells attaching the inner cell mass to the cytotrophoblastic shell. The shrinkage space to the left of the inner cell mass, seen in the last section, is here just closing in. The margin of this space next to the inner cell mass clearly consists of primitive mesoderm, and this is strong evidence that the appearance seen in figure 9 was correctly interpreted.

In proceeding to figure 11, there is apparently one section missing. We are therefore two sections deeper into the ovum and this, in reality, is the fourth section of the inner cell mass. It was a drawing of this section that Dr. Miller published in his original article. Owing to the low power of his drawing, one can not make out definitely the nature of the inner cell mass, and, misled by its appearance, I referred to it in a previous paper (Streeter, 1921) as consisting of a solid mass of ectoderm. Here, however, it is clear that it consists of an ectodermal vesicle—the amniotic vesicle. Within the amniotic cavity there is a cellular mass, apparently not lying free within the cavity but simply representing an edge of the wall, cut tangentially and bulging into the lumen. Apparently, at the outset the amniotic cavity is quite irregular in outline, as one might expect. The outer margin of the amniotic vesicle wall is irregular, particularly the upper border, i. e., that most distal to the body-stalk region. One must consider the possibility that these more loosely lying cells represent the endoderm cells separating themselves from the main inner cell mass.

As in the preceding sections, the inner cell mass is completely closed in by primitive mesoderm, and below the inner cell mass it can be seen how this primitive mesoderm forms the body-stalk, attaching the inner cell mass to the cytotrophoblastic shell.

By far the best view of the primordium of the embryo is shown in figure 12. Between this and the last section there appears to be a section missing, which would make it, in reality, the sixth section through the inner cell mass. Here one sees the thick-walled amniotic vesicle with a clear amniotic cavity. To the left and above, the wall is 2 to 3 cells thick, and this region apparently represents the future embryonic disc or shield. On the other hand, the thinner wall to the right is destined to form the amnion. If we search for endoderm or yolk-sac, two possibilities here present themselves. One may regard the looser cells along the upper and left margin of the amniotic vesicle as endoderm cells just detaching themselves. There is also to be considered the conspicuous triangular syncytial mass, lying to the left and above the amniotic vesicle and separated from it by a narrow cleft. This, I am inclined to believe, is the endoderm or yolk-sac mass. It is in correct position for a yolk-sac and its cytoplasm is stained intensely with eosin, which differentiates it from the surrounding primitive mesenchyme. It is prudent, however, to be on one's guard, as we are here dealing with a single section. The possibility of this being a tangential cut through a sheet of primitive mesoderm must be kept in mind. The next section or two below this would have revealed its nature.

Unfortunately, the succeeding three sections are missing. The section on slide 5 (fig. 8) is probably below the region of the inner cell mass, or, if there was any of it present, it has been torn away through mechanical injury. The identity of the yolk-sac must therefore remain an open question, to be determined by more perfect series of future specimens. Owing to the extreme rarity of human material, it is likely that our knowledge of the early history of the endoderm will have to come through the study of closely allied primate ova.

The primitive mesoderm in figure 12, as in the previous sections, everywhere surrounds the inner cell mass as a membranous envelope, at the same time attaching it to the trophoblastic shell, thereby constituting a primitive body-stalk. The triangular mass suspected of being the yolk-sac is included in a common compartment with the amniotic vesicle, there being no primitive mesodern in the cleft between them. It is in this cleft that the true mesoderm should later make its first appearance.

In 1923 and 1924 some letters were exchanged between Dr. Miller and Professor W. von Möllendorff, with reference to the publication of photographic reproductions of the Miller ovum in the Bildarchiv g. m. b. H. Freiburg/Br., which correspondence I have been permitted to read. From it I learned that the negatives were prepared for this purpose under the direction of Professor von Möllendorff, but, owing to post-war economic difficulties, the publications of that corporation have been suspended, with no definite prospect of resumption.

Before seeing the sections, von Möllendorff (1921) had referred to the Miller embryo and estimated its stage of development as being somewhat older than the Teacher-Bryce I embryo and younger than the Peters ovum, basing his judgment on the advanced differentiation of the primitive mesoderm, which he pointed out had already acquired distinct spaces between the trabeculæ. After seeing the sections he adhered to that view. Because of the importance of an opinion from so experienced an embryologist, I am quoting in free translation his observations as given in a recent paper (von Möllendorff, 1925, p. 29).

> My (original) interpretation of the Miller ovum is completely confirmed by the examination of the specimen, which the author kindly placed at my disposal. It is certainly an older ovum (than the Teacher-Bryce I); the questionable embryonal node represents a section through the ectodermal vesicle, apparently cut through the posterior part of the amniotic cavity and may well be a very early stage of development. One can not be sure of this, however, because, of the total series, only 5 sections passing through the implantation site are available. I agree entirely with Grosser that the structure of the trophoblast of this specimen must be reckoned as belonging among the youngest known.

ENDOMETRIUM.

Next in importance to the ovum itself are the various fragments of endometrium, in which one can observe the histology of the lining tissue of the uterus at the time of its penetration by the ovum. As can be seen in figure 16, the endometrium has the general characteristics of the premenstrual type. If one compare this photograph with Schröder's illustrations of the successive stages of the cycle, it resembles his twenty-sixth day of the regular 4-week cycle—that is, 2 days before the beginning of the next period (Schröder, 1922, fig. 41). Perhaps the irregularity and enlargement of the uterine glands in the Miller specimen are not quite so marked as in his case. The Miller endometrium resembles very closely the premenstrual mucous membrane shown by Hitschmann and Adler (1908, Taf. 5, fig. 3), in which the line of separation between compacta and spongiosa is already indicated and at which time the wide bulging glands of the deeper layers contain abundant secretion.

Of the different fragments of endometrium in the Miller specimen the one illustrated in figure 16 is cut most nearly transverse. The left end of the section is nearly longitudinal to the uterine glands and correctly represents the total thickness of the endometrium. In the specimen, near this region but beyond the part shown in the photograph, one can recognize the transition into the muscularis, strands of which stream upward between the glands. At the right end of the photograph, owing to the folding of the endometrium, the glands are cut obliquely. This exaggerates the width of the glands and also the thickness of the endometrium. In general, one can recognize, near the surface of the endometrium, a more compact area, consisting of edematous stroma, through which pass the mouths of the glands. Roughly, this comprises about one-eighth of the entire thickness of the endometrium, and it is in this superficial compact portion that the ovum is implanted. The surface of the compact layer is relatively smooth and is covered by a single layer of rather low cylindrical epithelium. A typical area of the surface epithelium is shown in figure 18. This, then, is the structure through which the ovum opens its way to imbed itself in the underlying stroma, either by merely crowding apart the epithelial cells and wedging its way into the stroma, or by eroding an opening in the epithelium sufficiently large to allow its passage. In some portions one sees evidences of secretory activity of these surface cells, but it is not nearly so marked as that shown by the epithelium of the glands. Here and there on the surface are a few cells that have beautiful cilia; in other places one can not be sure whether the surface is covered with cilia that are matted down, or with a layer of granular secretion products. For the most part the surface epithelium is devoid of cilia. Beneath the epithelium the stroma is quite edematous and in its meshes one finds a considerable amount of granular coagulum stained brilliantly pink by eosin. Some of these larger granules can be seen in figure 18. The finer ones do not show here nor in figure 16. Under the low powers the picture suggests extravasated blood, but under the higher it appears to be only coagulated serum, devoid of red cells. If one hunt carefully one finds in a few areas small masses of faded red cells, which seem to represent true extravasations. The stroma cells of the compact layer do not show as yet any decidual transformation, except in the region immediately surrounding the ovum, to which reference has been made. In this respect the Miller specimen differs from the Kleinhans specimen, described by Grosser (1922), who finds in that specimen beginning decidual reaction in all parts of the endometrium, in remote regions as pronounced as directly around the ovum.

In contrast to the relative quiescence of the surface epithelium, the epithelium of the uterine glands shows every evidence of active secretion. A typical gland is shown in figure 17. Owing to the proliferation of their cells, the epithelial walls are forced into irregular folds, so that in a longitudinal section of the glands they present the well-known saw-tooth appearance. In these sections, however, I can find no mitotic figures and it is evident that the proliferation stage has passed.

On examining the epithelium more in detail, one finds it in various stages of secretory activity. The greatest activity appears to be in the deeper lying portions of the glands, although there is some activity in the more superficial parts all the way to the outlet. Other than the greater activity of the deeper parts, I can find no definite distribution of the different stages of secretion. A single gland may show the different stages in close juxta-position. I have selected areas, photographs of which are reproduced in figures 19 to 22, which show typical stages of this phenomenon. The epithelium in figure 19 shows high cylindrical cells, the apical portions of which are filled with fine granules, separated from the lumen by a sharp margin. This appears to be typical of the cell just before the secretion emerges into the lumen. A group of cells of this type, as seen under lower magnification, is shown in figure 17, at the point marked by an asterisk. In the stroma surrounding the gland in figure 19, in addition to the spindle-shaped stroma cells, one can see a typical clasmatocyte. The stroma in general is edematous; that shown in figure 19 is more compact than in most regions. The stroma tends to be more compact immediately adjacent to the glandular epithelium. In figure 20 one can see what appear to be globules of the secretion substance extruding from the surface of the epithelium. These resemble the processes described by Corner in the surface epithelium of the pig uterus (Corner, 1921, plate 4, fig. 33), which may very well represent the same phenomenon. In none of the glands do I find in the protoplasm of the epithelium any well-marked vacuoles, such as are pictured by Schröder (1922, fig. 40a), either in the earlier or in the later stages of the secretory process. This is doubtless to be attributed to differences in fixation or the condition of the tissues.

A little later stage in this process of secretion is illustrated by the area shown in figure 21, where one finds the epithelium frayed out, with a considerable loss of protoplasm and with globules of secretion extending well into the lumen of the gland. In some places one finds the lumen entirely filled with this globular type of secretion. In other places it appears to have become broken up and arranged in a fine granular network. It is this material that shows the mucin reaction so beautifully pictured by Hitschmann and Adler and others. In some glands the secretion is coagulated in homogeneous islands. Sometimes embedded in these islands one can recognize the globular type still intact. This is illustrated in the area shown in figure 22, which is characteristic of the last stage of the secretion process. There then remain only the basal portions of the cells, which appear more spread out than in the earlier stages, suggesting that in this phenomenon some of the epithelial cells are entirely destroyed in the production of the secretion. In such areas one can clearly identify the membrana propria of the epithelium. These illustrations are sufficient to indicate the high degree of functional activity that is shown by the uterine glands at about the time the ovum implants itself. In the present case we are doubtless at the end of the secretory phase and the present gland content can be of no nutritive service to the ovum. In fact, in slightly older embryos one finds the glands undergoing rapid regression.

COMPARISON OF THE MILLER EMBRYO WITH OTHER YOUNG HUMAN EMBRYOS.

In studying the Mateer embryo a few years ago, and in attempting to place it in its proper position relative to embryos that had been described in the literature, it became clear to the writer that the factor of size of the chorion was of less significance than the degree of its differentiation and that of the embryo. The manner of treatment of an embryological specimen and the amount of its shrinkage and folding greatly affect the size, particularly as regards the dimensions of the chorion, this disproportion between size of chorion and development of embryo being even greater where pathological elements have entered. This is especially true for the latter part of the presomite period. On the other hand, in the very earliest stages, the size of the chorion appears to be a consistent index of the development of the ovum, and perhaps this is explained by the fact that the trophoblast and its derivatives are more precocious than the inner cell mass and that it is in those parts that the principal increase in size is then taking place.

In determining their proper seriation, I found it convenient to separate the embryos of the presomite period into three groups: (1) those specimens in which the primitive groove is not yet formed and which are consequently devoid of body mesoderm; (2) those in which the primitive groove is present; and (3) those having, in addition to the primitive groove, a neural groove and its associate structures. In addition, Group I was subdivided into: Stage 1, those in which there were as yet no chorionic villi; stage 2, those in which primitive villi could be recognized; and stage 3, those in which the villi had become branched. Subsequently, other groupings of these early embryos, based on structural differentiation, have been made by von Möllendorff (1921) and Bryce (1924), and I think with improvement over the classification suggested by me. They both used the differentiation of the inner cell mass as the principal criterion for all their stages, whereas my subdivisions of Group I were made on the basis of the structure of the chorion. In the present instance we need consider only those embryos in which there are as yet no chorionic villi—that is, my stage 1 and Groups A and B of Bryce. In addition to the Miller specimen there are thus far known only three embryos that clearly belong to this period; these are the Kleinhans (Grosser), von Möllendorff Sch., and Teacher-Bryce I.

For our knowledge concerning the Kleinhans ovum we are principally indebted to the excellent description of Professor Grosser (1922). The specimen was given to him by Professor Kleinhans, who originally made the sections and some years later discovered the presence of the ovum. Only one of the sections that were saved passes through the ovum, and moreover, this section is well toward one side, so that neither the inner cell mass nor any of the primitive mesoderm is shown. However, it does present a good picture of the early trophoblast and the site of implantation. Through the kindness of Professor Grosser, the Carnegie Embryological Laboratory has been favored by the gift of a series of excellent photographic negatives made by him and showing in sharp detail the structure of the trophoblast in selected areas. A comparison of these negatives and Grosser's illustrations with the sections of the Miller embryo reveals striking similarities between the two ova. The differences that exist are consistent if one assume that the Kleinhans specimen is a little older than the Miller ovum, a conclusion that has been reached by Grosser (1922), Teacher (1924), and Bryce (1924). In the two specimens the cytotrophoblast forms much the same sort of irregularly kinked wall, made up of a single layer of cuboidal cells. Proceeding outward one finds the plasmoditrophoblast or syncytium arising from the outer surface of the cytotrophoblast with the transitional zone cut in various planes. As in the Miller specimen, it is characteristic of the transitional syncytium to exhibit nuclei with clear areas partially surrounding them, these clear areas representing the beginning lacunæ, or in some cases recesses from adjacent lacunæ. The more mature syncytium lies farther out, and in the Kleinhans specimen one finds it more extensively spun out than in the Miller, and consequently larger lacunæ are produced with relatively more slender syncytial strands outlining them. The still more striking and chief difference between the two ova is the fact that the lacunæ are now well filled with blood, particularly in the basal portions of the ovum. The red cells are sharply outlined, and among them is an unduly large number of leucocytes, which might be expected of stagnant blood. Along the outer margin of the trophoblast there is an abrupt transition to the maternal tissue, which, except for being mechanically crowded outward, shows little effect due to the presence of the ovum. In the immediate neighborhood there is a moderate invasion of leucocytes, as was noted in the Miller ovum, which must be interpreted as a reaction to the presence of the ovum. In addition to this, some of the stroma cells begin to resemble decidual cells, but no more so than is seen in the Miller ovum. Finally, as to the endometrium as a whole, one may best judge by comparing my figure 16 (plate 5) with Grosser's (1922) figure 1. The resemblance is very close. The separation of the compacta from the spongiosa appears a little more pronounced in the Kleinhans specimen, but this may be because the section was cut thicker. I find no statement as to the thickness of the section. If one may judge from the epithelium of the uterine glands, it is considerably thicker than the sections of the Miller ovum. The character of the uterine glands in the two specimens is essentially the same.

Coming to the embryo Sch., described by von Möllendorff (1921), one is immediately impressed by its general resemblance to the Teacher-Bryce I, and it must represent about the same stage of development. The resemblance lies chiefly in the form of the syncytial trophoblast and the character of the implantation site. The inner cell mass, or embryo proper, and the form of the chorionic cavity do not correspond satisfactorily to their counterparts in the Teacher-Bryce I. In fact, they depart so far from what is seen in the other ova of this group and those in the next older group that for the present one is warranted in regarding this specimen with suspicion. Judging from the illustrations, its tissues leave much to be desired in their histology and show evidence either of degeneration or poor preservation. On this account a further comparison will not now be attempted. The interested reader is referred to von Möllendorff's carefully prepared description.

The Miller specimen represents the stage of formation of the trophoblastic lacunæ. The Kleinhans represents the stage at which these syncytial lacunæ become flooded with blood. The Teacher-Bryce I (and probably also the von Möllendorff Sch.) represents the stage at which the blood-saturated syncytium encroaches on the surrounding maternal tissues, producing a narrow necrotic zone adjacent to the enlarging ovum, beyond which zone the endometrium is distinctly decidual in character. These three ova thus represent successive stages in this early period of implantation.

The detailed structure of the Teacher-Bryce I is now so well known, through the complete and well-illustrated accounts given by those authors (Bryce and Teacher, 1908; Bryce, 1924; Teacher, 1924; Teacher, 1925), that short reference here will suffice. I would only call attention to figure 2 (plate II) in the 1908 monograph. This excellent photograph was taken by Professor Teacher and gives one of the best views of the implantation site. One can see that, compared with the Kleinhans specimen, the syncytium is more abundant and spun out in thinner strands. On the one hand, it is still actively arising from the cytotrophoblast, and on the other, ends rather abruptly against the maternal tissues, being everywhere surrounded and filled with blood. Where the syncytium comes in contact with the maternal tissue, the latter shows marked necrosis and one sees many macrophages lying amidst the disappearing tissue. These appear to be monocytes that have wandered out from the stagnant maternal blood of the syncytium and have become large and prominent from the abundant nourishment at their disposal. Outside of the necrotic zone the endometrium is distinctly decidual in character. This picture of the mechanism of the excavation of the implantation cavity is perhaps the most important feature of this famous embryo. It is true that the specimen was aborted spontaneously and the tissues show some maceration, but one must accept it as essentially normal, since it harmonizes completely with the younger and older stages. The imperfect histological character of the material is less serious in the case of the trophoblast than in the case of the primitive mesoderm and the derivatives of the inner cell mass, where one would wish for better preservation of the tissue. The primitive mesoderm is more abundant than in the Miller ovum, but one can not be sure of its arrangement. We would expect a more definite membrane underlying the cytotrophoblast. Perhaps the whole thickness of the mesoderm constitutes such a membrane, but in this case swollen and edematous, leaving a constricted tissue-cavity at the center. The amniotic vesicle and yolk-sac should be ensheathed more or less by the primitive mesoderm, and in such a way as to provide a body-stalk, the future amnion pressing against the chorionic membrane. The separation of the amniotic and yolk vesicles must also be accounted for. Maceration of the primitive mesoderm would explain all of this. With these adjustments taken care of, one could pass on, without any serious gap, to the solid ground of the Peters embryo.

Concerning illustrations, I should mention that the photographs shown on the accompanying plates were made by Mr. O. O. Heard, a member of the staff of this laboratory. The faithful manner in which they portray the structure of this important embryo is to be credited to his skill and the pains taken by him in their preparation. The colored illustrations shown on plates 7 and 8 were photographed directly from the specimen with the use of suitable color filters and a superimposed half-tone screen. The negatives thus obtained were transferred and printed from stone, under the personal direction of Mr. A. B. Hoen, without whose help and advice this manner of reproduction could not have been carried out.

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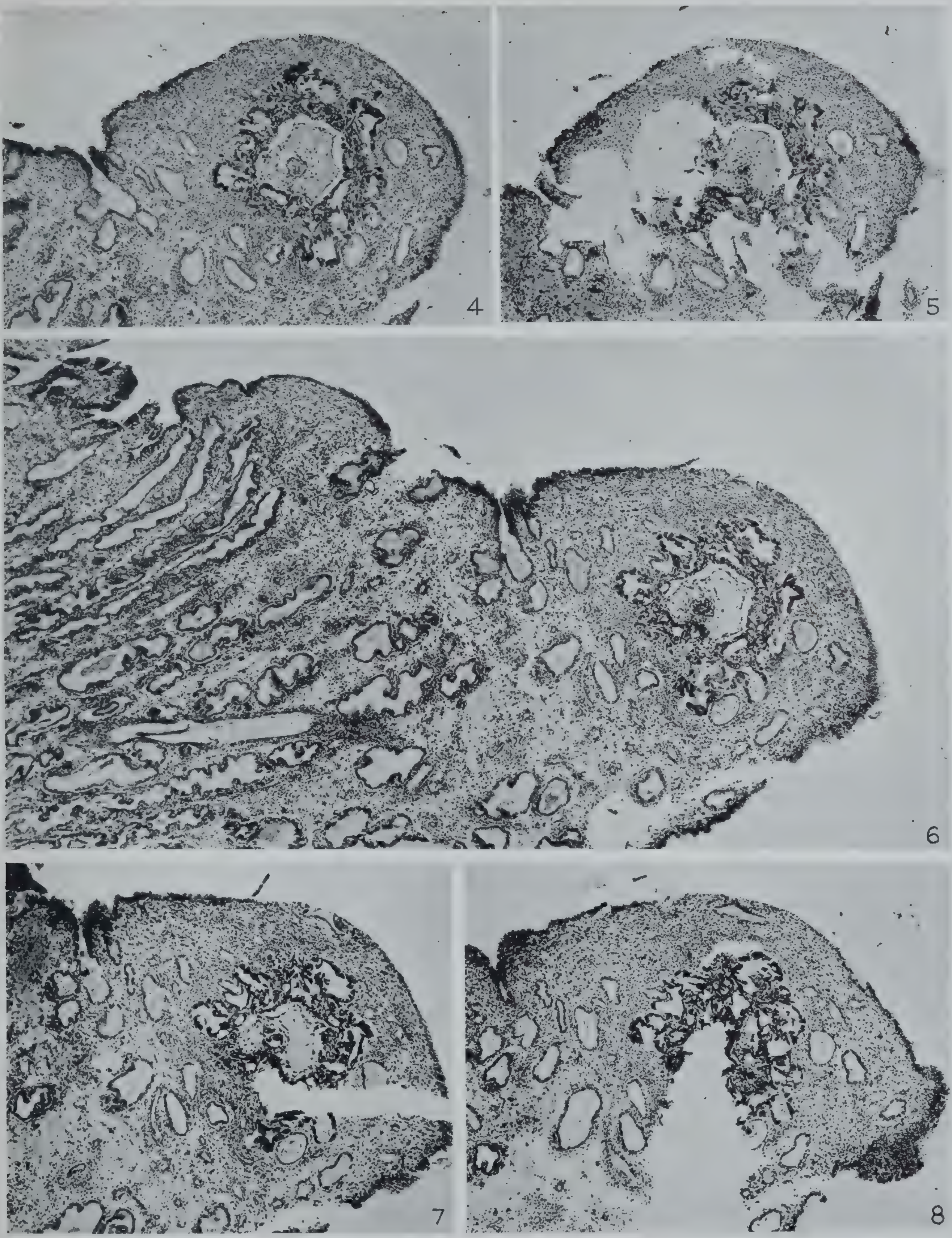

The figures on this plate are intended to show the general topography of the ovum and surrounding endometrium. The sections are cut tangentially through the summit of the implantation site. They then pass through the tip of the fragment containing the ovum and are arranged in sequence, beginning with the most superficial and ending with the one through the most deeply lying part of the ovum. ×40.

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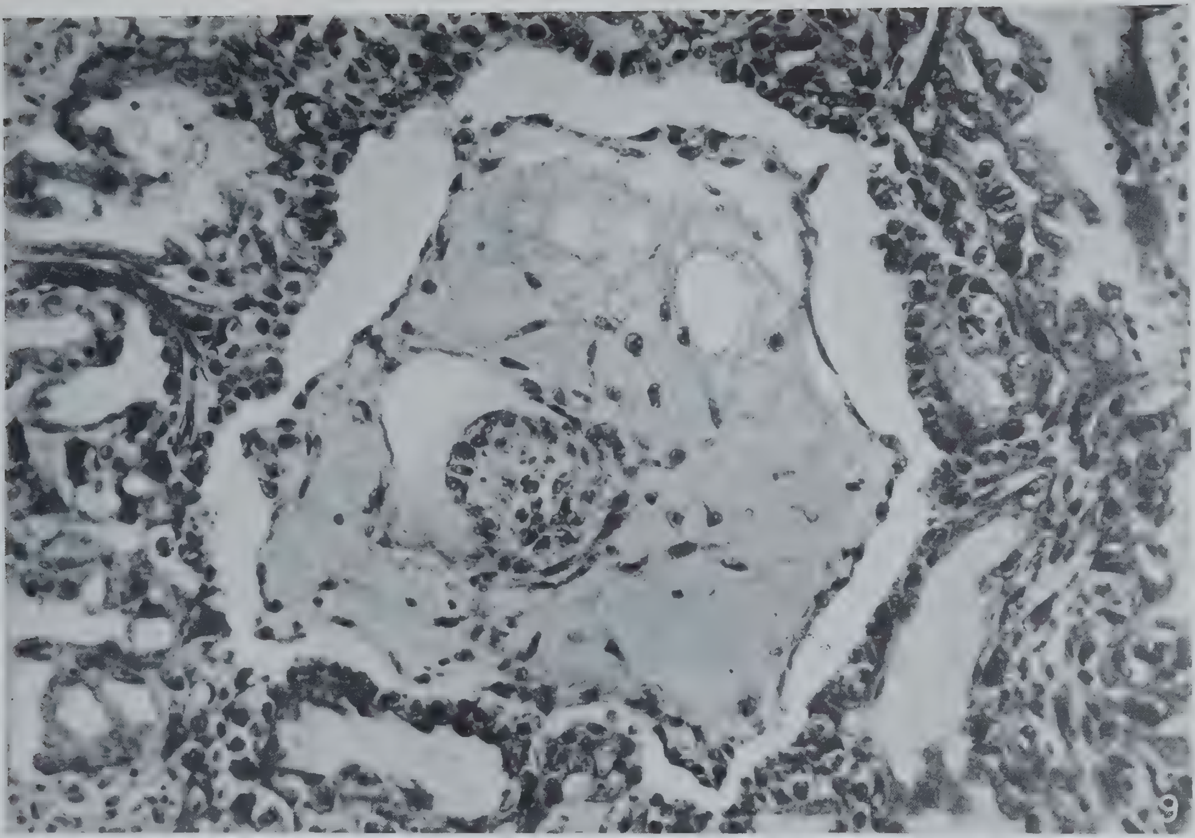

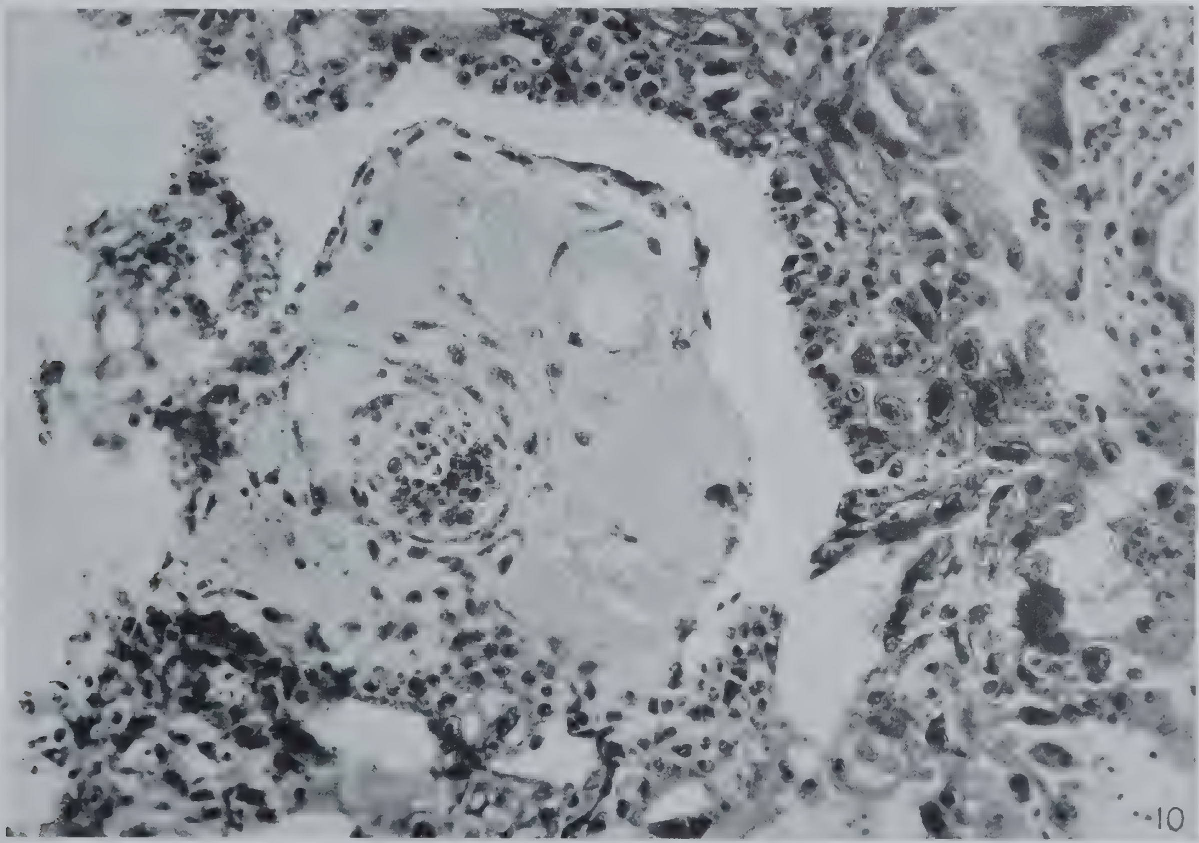

Figs. 9 and 10 are in series with the figures on the next plate, showing the detailed anatomy of the cavity of the ovum with its inner cell mass. The cavity is closed in by the cytotrophoblast, which in turn is lined with primitive mesoderm, the two being separated by a shrinkage space. In addition to constituting a lining for the trophoblastic shell, the meshes of the primitive mesoderm fill out the cavity of the ovum and also inclose the inner cell mass and form the primitive body-stalk. × 250.

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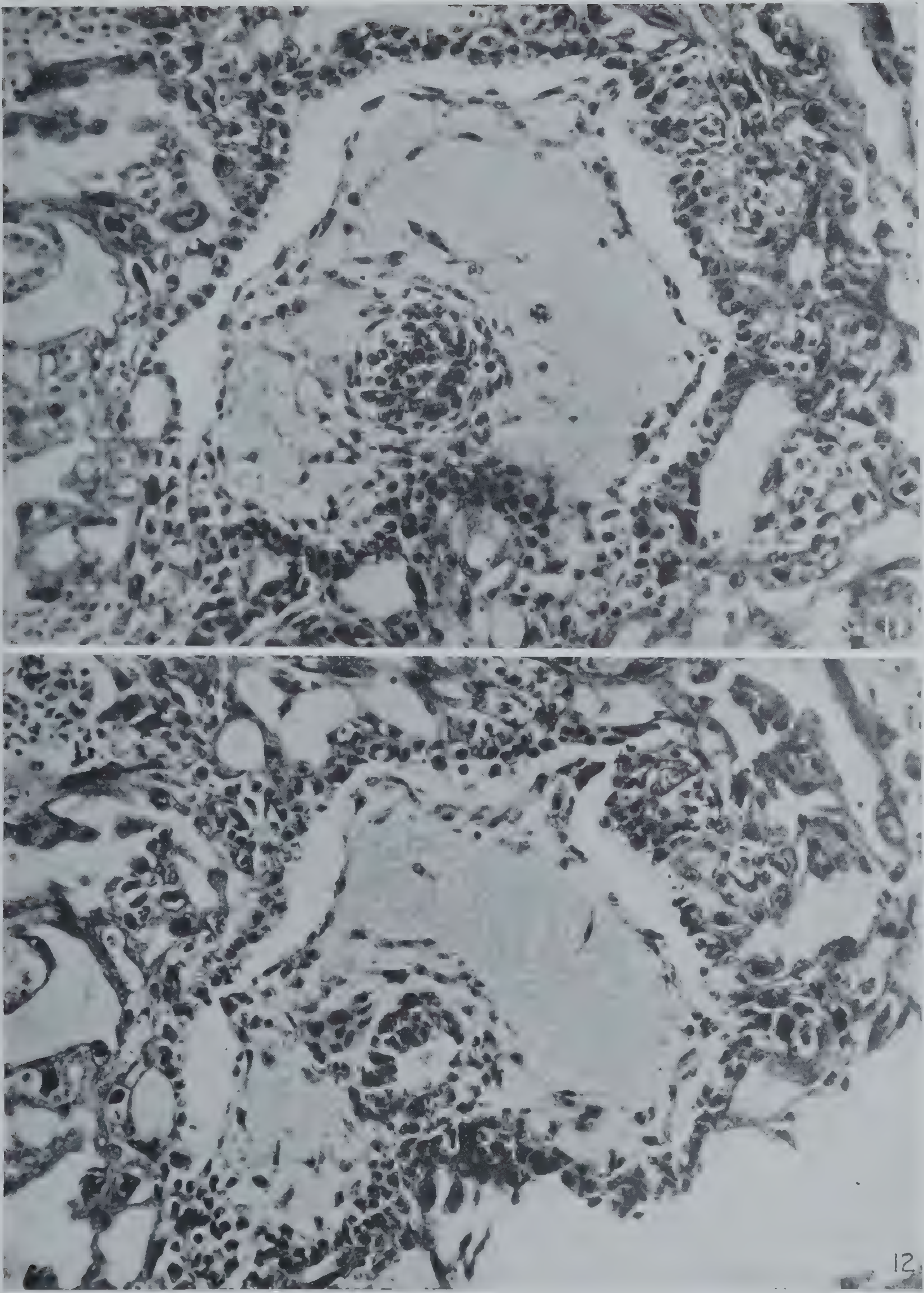

FIGS. 11 and 12 are from slides 3 and 4 and show enlarged views of the embryo. The best is in figure 12, where one can see a definite amniotic vesicle and, to the left, a mass of cells, apparently representing the yolk-sac. Many details of the structure of the trophoblast can be seen in these figures, but further details of the trophoblastic fringe are shown on plate 4. × 250.

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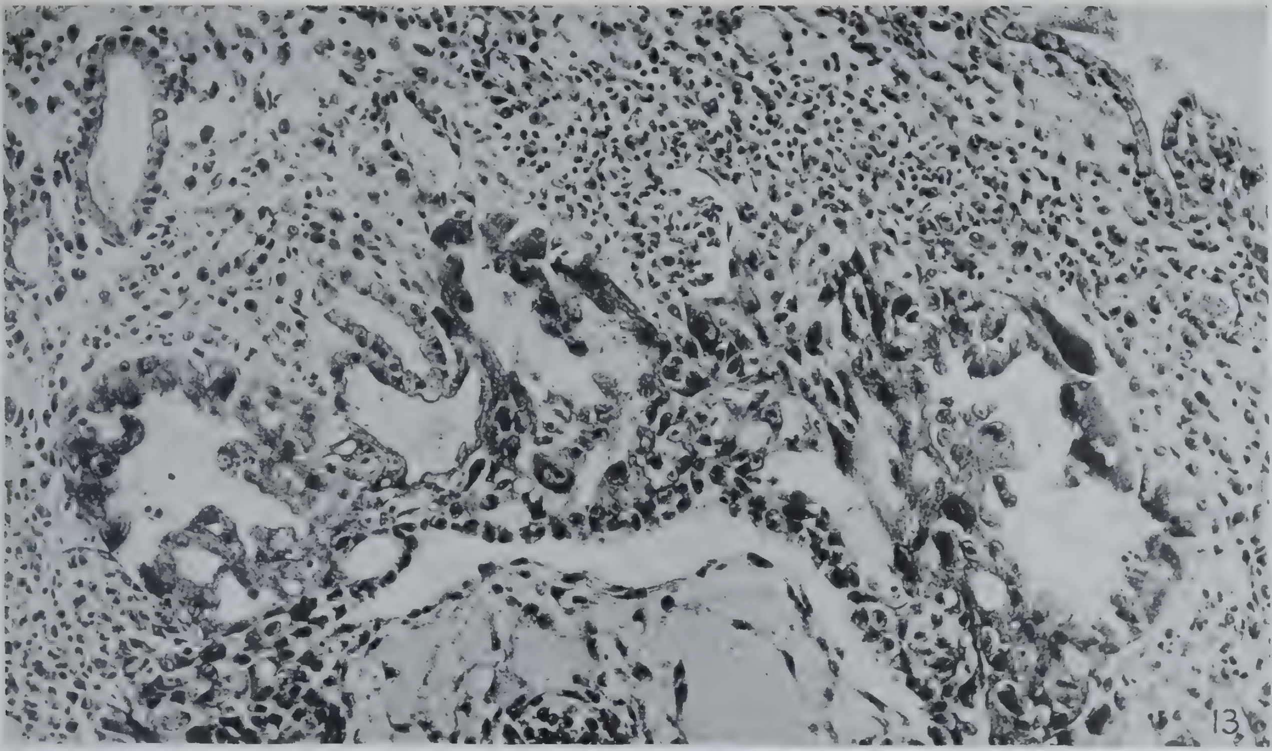

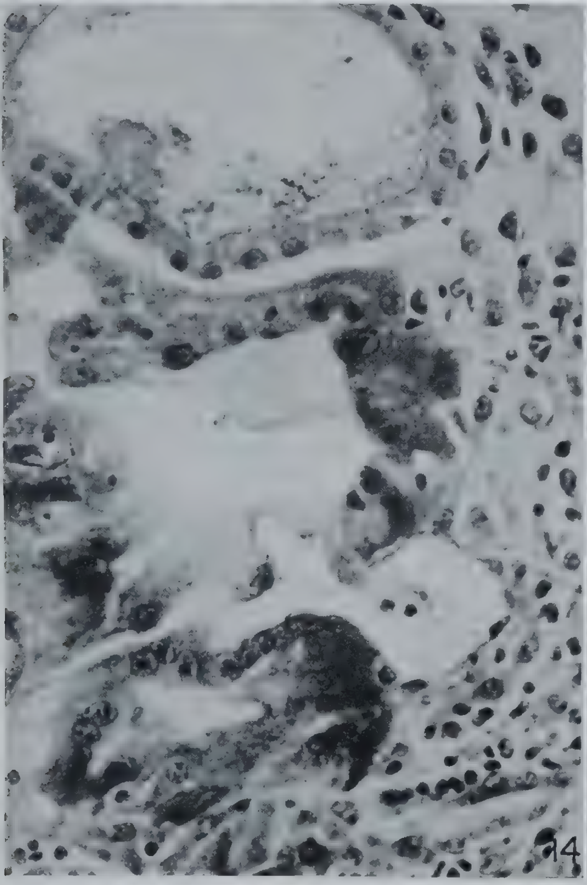

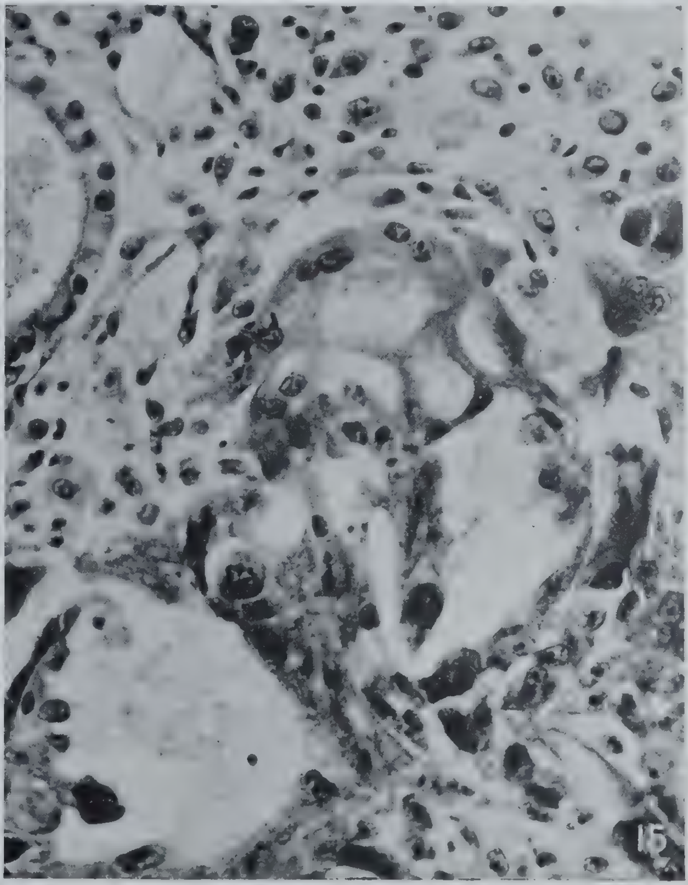

Fig. 13. Photograph of a part of the trophoblastic shell, taken from slide 3, showing the cytotrophoblast and its transition into the surrounding plasmoditrophoblast, beyond which lies the endometrial stroma and uterine glands. ×200.

Fig. 14. A portion of the trophoblastic syncytium that has opened a capillary, allowing free communication between the maternal blood system and the lacunæ. A uterine gland is shown above. ×400.

Fig. 15. A portion of the trophoblastic syncytium, showing its detailed structure and the character of the surrounding stroma. It will be noted that some of the stroma cells show an increased amount of cytoplasm and are beginning to take on the form of decidual cells. Above and to left, edge of a uterine gland. ×400.

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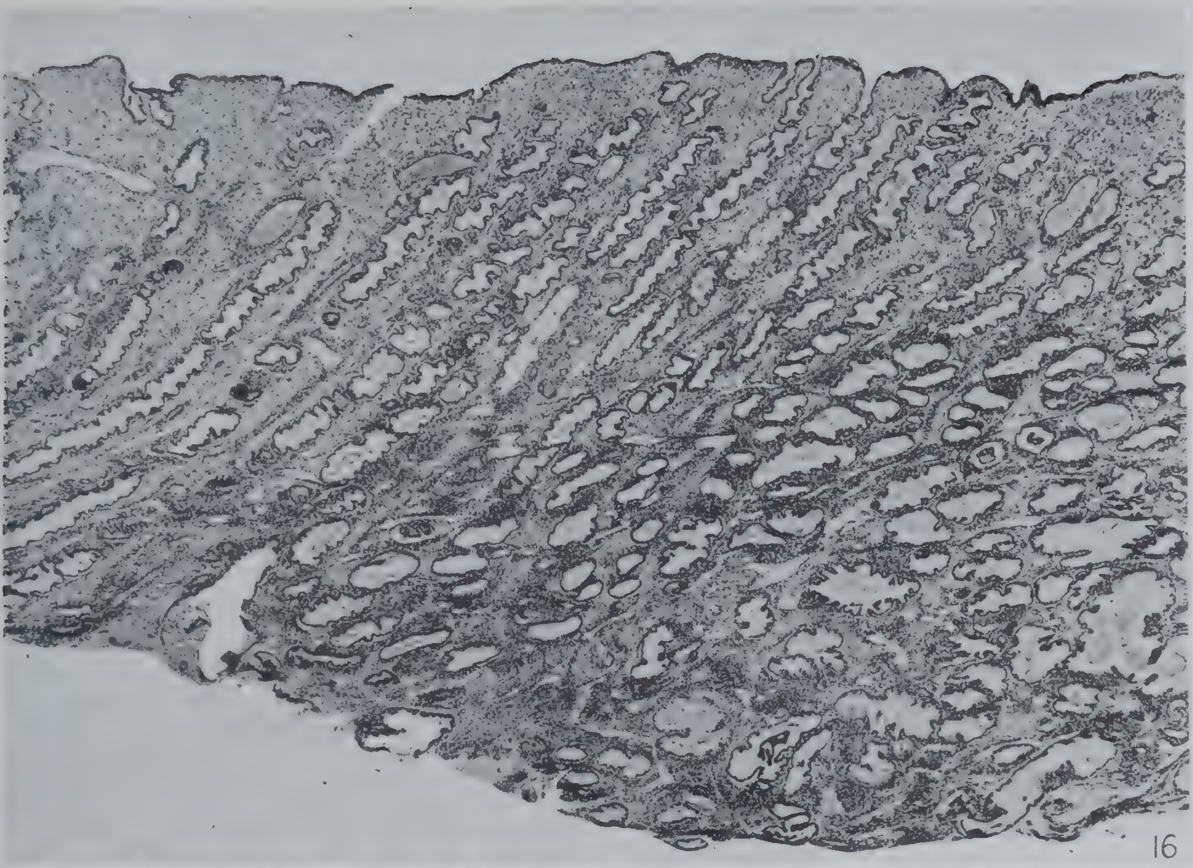

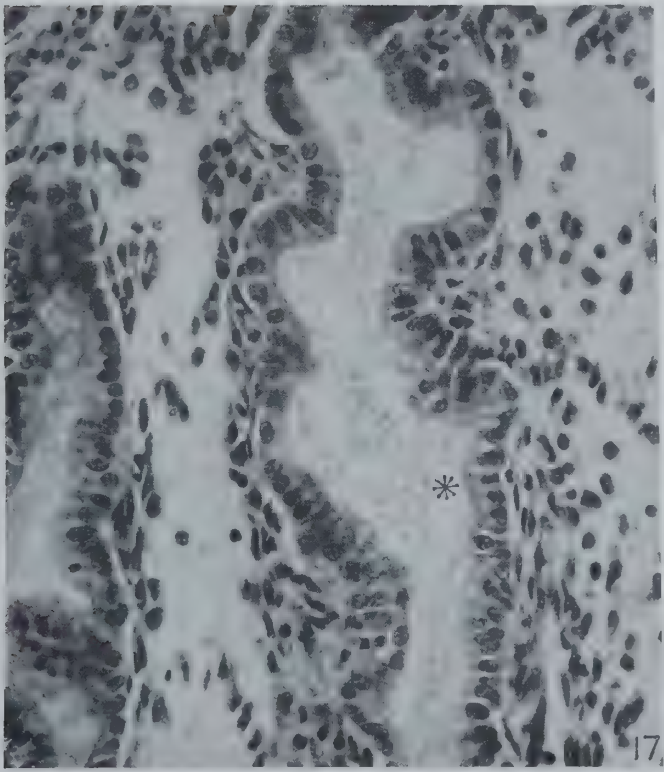

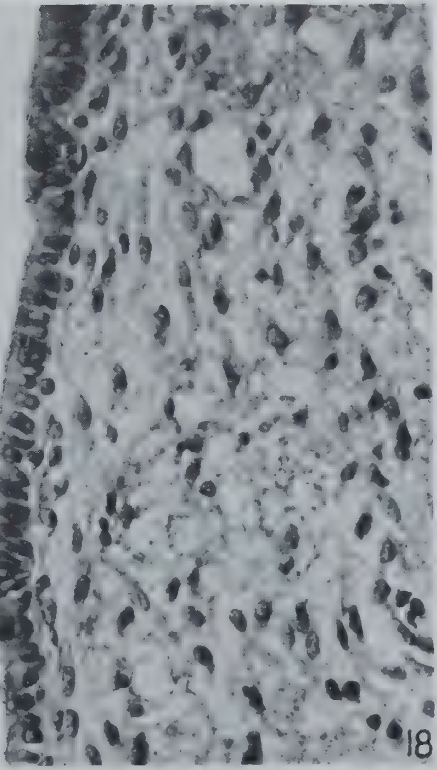

FIG. 16. Low-power view of the endometrium, showing its typical premenstrual character. Only the upper left-hand portion of the section is cut transverse to the surface, and that part correctly represents the thickness of the endometrium and its separation into compact and spongy layers. ×30.

FIG. 17. Showing the corrugated folding of the walls of a uterine gland and the edematous character of the surrounding stroma. ×400.

FIG. 18. Surface epithelium of the endometrium. Underlying stroma of the compacta is very edematous. ×400.

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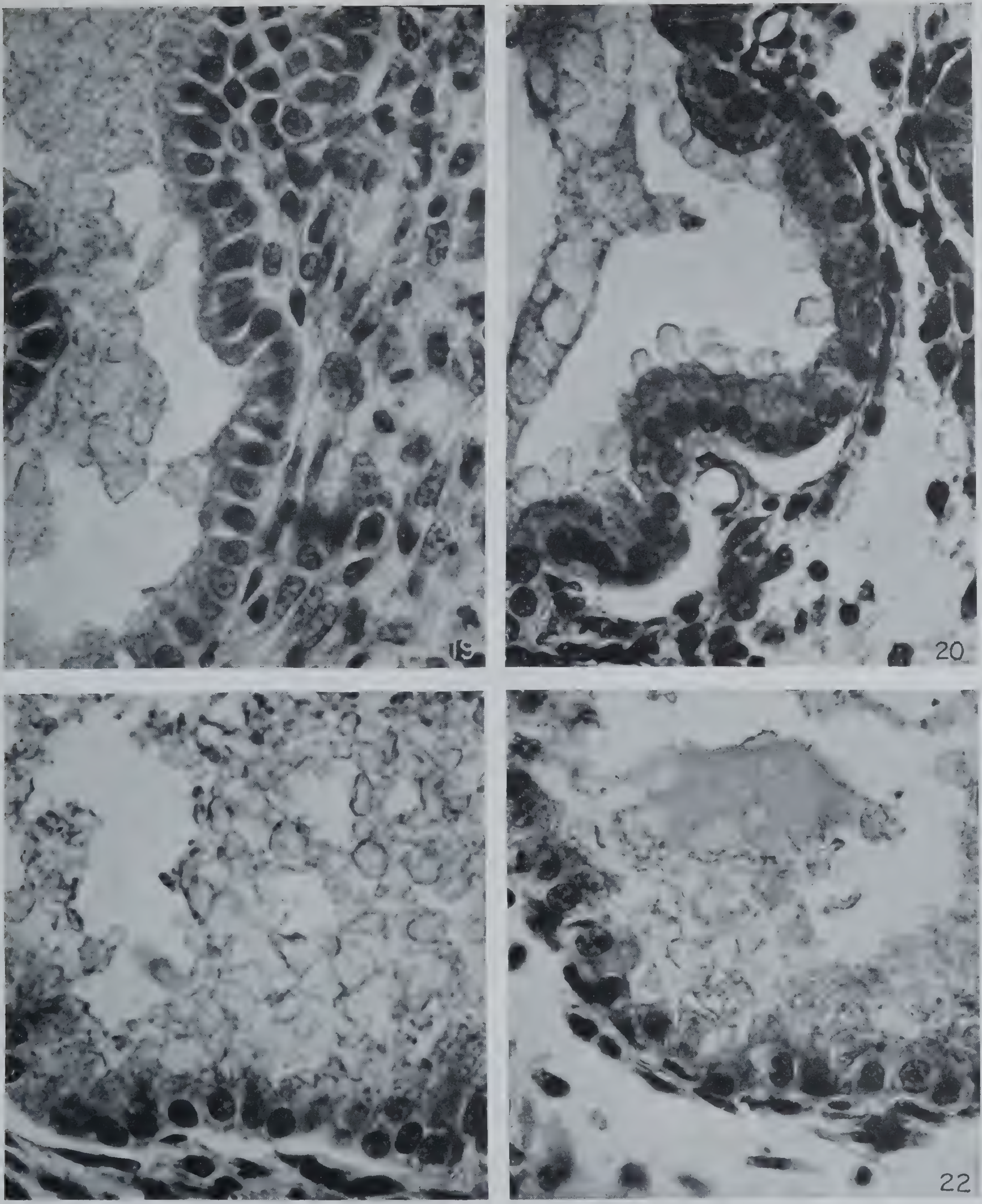

Figs. 19 to 22. Photographs showing four different stages in the secretory activity of the glandular epithelium. In figure 19 the apical portion of the epithelium is crowded with a fine granular mass preparatory to its discharge into the lumen of the gland; ×800. (This phenomenon is also illustrated in figure 17 at the point marked by an asterisk.) Figure 20, the discharge of substance from the epithelium into the lumen in the form of droplets; ×800. Figure 21, a more advanced stage of secretion, with fraying out of the apical portion of the epithelial cells; ×800. Contents of uterine glands often show this appearance, in which the form of secretion droplets is maintained. Figure 22, an advanced stage of secretion in which a considerable amount of epithelial protoplasm appears to have been lost in the secretion process; ×800. Photograph shows in the lumen an island of homogeneous coagulum, such as is frequently found in the glands. One sometimes finds imbedded in these islands a few secretion globules.

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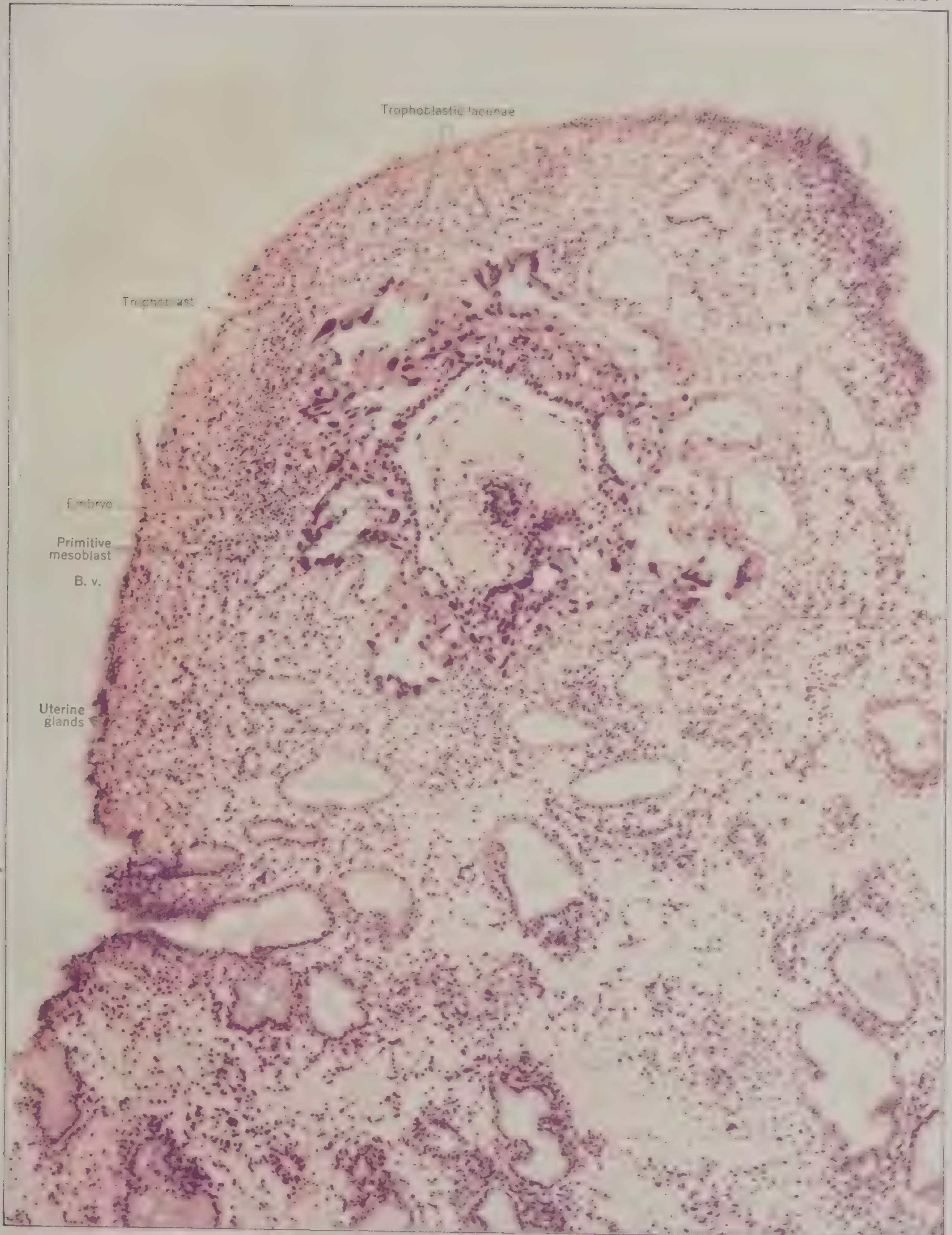

FIG. 23. Slide 3 of the Miller ovum, showing its general appearance in color and its relation to the surrounding endometrium. ×100.

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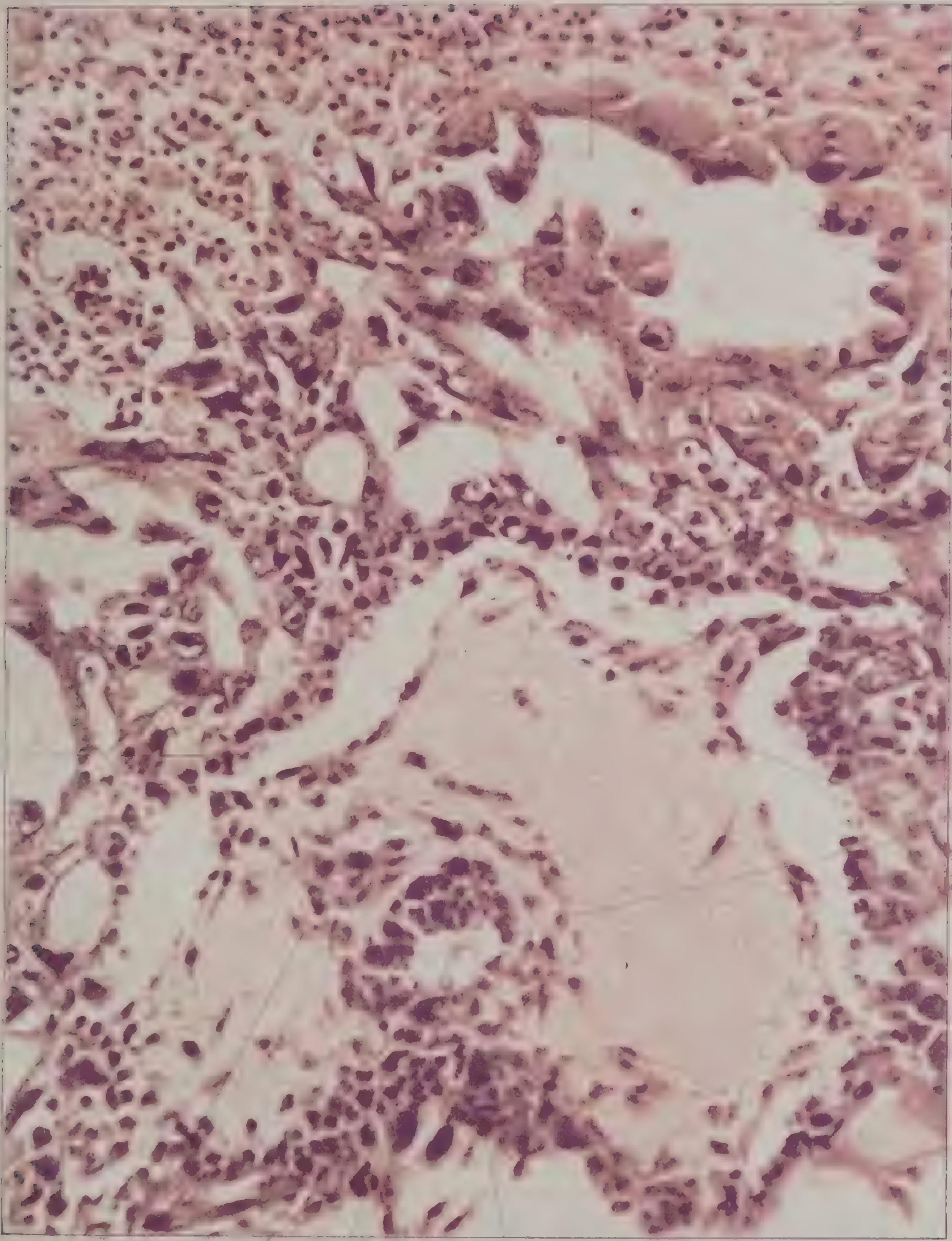

FIG. 24. Slide 4 of the Miller ovum, showing the amniotic vesicle and what appears to be a portion of the yolk sac to the left and slightly above. Surrounding these and filling in the cavity of the chorion is the primitive mesenchyme. Its meshes are filled with coagulated plasma. Primitive mesenchyme lines the trophoblastic shell, from which it has shrunken away, leaving a narrow artificial cleft. ×350.

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CONTRIBUTIONS TO EMBRYOLOGY, No. 93.

DETAILED FORM OF THE WOLFFIAN BODY IN HUMAN EMBRYOS OF THE FIRST EIGHT WEEKS.

By Jujiro Shikinami

(Okayama Medical College, Japan)

From the Carnegie Laboratory of Embryology, Baltimore, Maryland.

With seven plates.

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DETAILED FORM OF THE WOLFFIAN BODY IN HUMAN EMBRYOS OF THE FIRST EIGHT WEEKS.

INTRODUCTION.

If one wishes to become acquainted with the structure and form of any organ, there is perhaps no better way than to trace its development, step by step, back into the early embryonic stages. From such a groundwork the significance of its morphology and topography becomes apparent and, furthermore, only in this way can one hope to obtain a basis for satisfactory explanations of anomalous types and the various forms of faulty development that are often met with in all parts of the body. The need for an intimate acquaintance with all the steps of development is particularly important in the case of the organs of reproduction, because here we have not only to deal with an intricate system, but we also meet with marked differences in the two sexes. Much, of course, has already been done on the early stages of these organs. There is, however, a considerable gap between the known early stages and the mature structures. It is with the purpose of partially filling in that gap that I have undertaken a careful three-dimension study of the wolffian bodies, the results of which will be detailed in the following pages.

Owing to the intricate character of the tubular system of the wolffian body, there has always been difficulty in preparing an adequately illustrated account of it. Thus far, no paper has attempted to represent this organ in a way other than by schema or by outlines obtained through the use of the camera lucida. Although some authors have in that way obtained useful and fairly accurate diagrams, they have never attempted to present more than a general outline and the number of tubules. Furthermore, very few of these diagrams adequately show the relation of the wolffian bodies to other organs and the general contour of the body. It is therefore with much satisfaction that I take advantage of this opportunity to present carefully executed plastic illustrations, showing the morphology of the mesonephric units in a series of human embryos, ranging from 4 to 23 mm. in length. No attempt is made to study the finer histology of these organs, but reference will be made to certain histological features in connection with the determination of areas of growth and atrophy, as identified in the various stages.

Before beginning the description of the individual stages, I wish to express my appreciation of the opportunity given me by Professor G. L. Streeter for studying the specimens in the Carnegie Embryological Collection. It also gives me great pleasure to extend my thanks to Mr. O. O. Heard and Mr. E. Rever for their assistance in the process of making the reconstructions and, finally, I wish to acknowledge especially my appreciation of the assistance of Mr. James F. Didusch, who made the illustrations accompanying this paper and kindly assisted me in its translation.

MATERIAL AND METHODS.

A series of six human embryos was selected for the purpose of reconstruction. The numbers, sizes, and detailed information concerning these are given under the description of the individual stages. Other series of intervening stages were studied for comparison with these. It was found helpful to compare embryos of the same stage, cut in various planes.

The method of reconstruction is based on the Born principle, but in the Carnegie Embryological Laboratory many improvements have been instituted, which result in more perfect and complete models. The new method was described in considerable detail by Lewis[1] but it may here be explained in brief that the sections are either photographed serially on bromide paper or are traced with the projection apparatus on white paper at 50 or 100 diameters. The outlines are then transferred by means of carbon paper to wax plates of appropriate thickness and the portion traced is cut out from each plate and the plates piled so as to constitute a mold. In the cavity of this mold plaster of Paris is poured. When the plaster is sufficiently set the mass is suspended in hot water, which melts off the wax, leaving a plaster mold of the desired magnification. By this method 6 models were made, 4 of which were made by the author and 2 (Nos. 2 and 3) by Mr. Heard.

DESCRIPTION OF INDIVIDUAL STAGES.

STAGE I.

(Embryo No. 1062, 4.5 mm. G. L. Sections 20μ, transverse.)

There are present in this embryo 26 paired somites. The two wolffian bodies lie 0.55 mm. apart at their cranial extremities and 0.3 mm. at their caudal extremities. Both extend from the level of the ninth to the level of the twenty-fifth somite. The left is 2.43 mm. long (linear) and consists of 27 developing tubules. The first tubule is the smallest; it is pear-shaped but slightly curved, and its position is ventral to the primary excretory duct, with which it is not connected. Tubules 2 to 12 inclusive are also pear-shaped, are larger than the first and communicate with the primary excretory duct. Tubules 13 to 27 inclusive are vesicular in form and have not as yet become united with the duct. The twenty-seventh is attached to the mesonephrogenic cord by a small pedicle, the process of differentiation not having been completed.

The mesonephrogenic cord is 0.378 mm. long, cylindrical in form, with a slight uneveness of surface, its curvature parallelling that of the primary excretory duct. The duct is 2.75 mm. long, its curvature, like that of the entire wolffian body, conforming to the curve of the body of the embryo. The tubules forming the upper third of the mesonephros occupy a position relatively ventral to the duct, those of the middle third are situated slightly ventro-mesially, while the group forming the lower third lies mesio-ventral to the duct. No trace of a metanephros could be recognized in this embryo.

The right mesonephros is 2.31 mm. long (linear) and contains 26 developing tubules. Their positions relative to the mesonephric duct are essentially the same as those of the left mesonephros. Tubules 1 to 16 inclusive are pear-shaped and slightly curved, and are connected with the primary excretory duct. The remaining canals are vesicular in form and connections with the duct have not yet been established. The mesonephrogenic cord is 0.39 mm. long and its form is like that of the left cord, described above.

Alimentary Canal.—When viewed laterally, the curvature of the alimentary canal, like that of the wolffian bodies, very nearly parallels the curve of the body of the embryo. That portion of the intestine to which the yolk-stalk is attached is curved slightly in a ventral direction. Viewed ventrally, the alimentary canal, from the caudal end of the stomach to a point slightly below the attachment of the yolk-stalk, curves from left to right in the form of a shallow reversed letter S. The middle of the lower curve forms the point of attachment of the yolk-stalk. From the lower end of the reversed S-curve to its caudal extremity, the canal occupies a midline position in the body of the embryo, the tail-gut curving with the tail to the right. The stomach primordium appears as a ventral bulging of the alimentary canal; it is flattened laterally and is separated from the alimentary canal by a cranial and a caudal constriction, the maximum bulging occurring directly over the latter. The dorsal pancreas is represented as an out-pouching of the dorsal wall of the canal directly below the level of the caudal end of the stomach. On a line with the lower level of the pancreatic bulge the ventral surface of the alimentary canal shows two saccules, which are connected with it by means of a short pedicle, broad cranio-caudally and constricted laterally. In this pedicle a short, laterally flattened oval lumen connects the cavities of the saccules with the lumen of the canal. The upper, larger saccule, with its trabecular outgrowths, is the hepatic diverticulum, while the lower one is the primordium of the gall bladder. Viewed ventrally, the hepatic diverticulum bulges laterally. Its ventral surface is slightly flattened. No evidence of a ventral pancreas can be discovered. Directly below the rudimentary gall bladder, and attached to the ventral surface of the gut, is seen the vitelline duct, which is broad cranio-caudally and flattened laterally (its cranio-caudal dimension being 0.38 mm. and its lateral dimension 0.10 mm. at the widest point) and curves somewhat to the right. From the lower border of the point of attachment of the vitelline duct to the cloaca the intestine becomes progressively larger in size, maintaining the lateral flattening.

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[1] Lewis, W. H. The use of the guide planes and plaster of Paris for reconstruction from serial sections. Some points on reconstruction. Anat. Record, vol. 9, pp. 719–729, 1915.

The flattened, funnel-shaped allantois, at the point where it opens into the gut, occupies an area extending from the urorectal notch to a point close to the attachment of the mesonephric duct to the cloaca. There is evidence of a differentiation between the urogenital sinus and the rectum in the form of a shallow groove, which extends from the urorectal notch to the tail gut on both right and left side of the cloaca. Due to the pressure of adjacent structures, the tail gut is compressed laterally, causing a deep groove to be formed on its right side, extending almost its entire length and running parallel to its dorsal contour. Owing to the curve of the tail of the embryo to the right, the tissues are not so crowded on the left side, and pressure on the lateral surface of the gut being much less, the groove on this side is not nearly so deep.

STAGE II.

(Embryo No. 836, 4 mm. G. L. Sections 15μ, transverse. Figure 1.)

The length measurement of this embryo is less than that of the one just described because of its greater body curvature. The more advanced development of the separate organs determined the order in which these two specimens are described.

This embryo contains 30 paired somites. The left mesonephros extends from the lower level of the eighth to the lower level of the twenty-sixth somite. It measures 2.13 mm. in length (linear) and contains 33 developing tubules. Its cranial tip is opposite the level of the vitello-intestinal junction and its caudal end on a level with the center of the cloaca.

The first, and smallest, tubule is ventro-mesial to the mesonephric duct and can be divided into two parts—a tubular (the principal canal) and a bulbous glomerular portion (fig. 11). The tubule is connected with the wall of the mesonephric duct, but its narrow lumen does not communicate with that of the duct. The enlarged distal end of the lumen, together with a small cavity directly above it, constitute the lumina of the primitive glomeruli. The positions of the tubules are virtually the same as in the specimen described under stage I: viz, from ventro-mesial in the cranial end to frankly mesial in the caudal end of the mesonephros, in relation to the position of the wolffian duct. Tubules 2 to 10 inclusive, with the exception of the seventh, are S-shaped. Tubule 7 is made up of a short tubular portion and an enlarged, flattened vesicular end, in which there is a wide lumen with a narrow extension into the tubular portion. Tubules 2 to 7 are attached to the wall of the mesonephric duct, but communication between their lumina and the lumen of the duct has not yet been established. In tubules 8 to 11 such communications can be seen. In shape and diameter the lumina conform in a general way to the tubules; those of the S-shaped tubules exhibit the S-curves; in tubules with a simple bend the lumen follows that bend, and in the vesicular tubules the lumina are round or oval cavities. In tubules 1, 5, 12, 15, 16, 22, 23 and 28 there are two lumina. Tubules 12 to 21 inclusive and tubule 31 are attached to the wall of the mesonephric duct but their lumina do not communicate with the lumen of the duct. The tubules are considerably crowded.

Caudal to the thirty-third tubule is the mesonephrogenic cord, a cylindrical cell mass ending in a blunt point. Its length is 0.27 mm. and it contains three irregularly formed cavities, the one in the cranial portion being the largest, while the one in the caudal region is the smallest. The surface modelling of the cord, like that in the first stage described, is slightly uneven. When viewed laterally, the curvature of the mesonephros, with its tubules and primary duct, nearly parallels that of the body of the embryo, the cranial end swerving slightly from the parallel course in a ventral direction. The left primary excretory duct, which is 2.86 mm. long, presents a slight enlargement at its cranial tip, becoming progressively narrower in a caudal direction, then broad and laterally compressed in its middle third, again gradually narrowing as it approaches the cloaca. Directly at the point of its attachment to the cloaca appears a slight bulbous enlargement. The duct shows throughout its length slight variations in size. No trace of a metanephros could be discovered in this embryo.

The right mesonephros extends from the middle of the eighth to the lower level of the twenty-seventh somite; it is slightly longer than the left, its length being 2.55 mm., and contains 34 developing tubules. The first tubule is the smallest and consists of a short tubular and a small bulbous glomerular portion. Its proximal end is attached to the mesial surface of the primary excretory duct, its distal glomerular portion lying in a position ventral to that of the duct. The positions of the tubules in relation to the duct are the same as those of the right side, viz, from ventral in the cranial portion gradually to mesial in the caudal end of the mesonephros. Tubules 2 to 14, with the exception of tubule 11, are in the form of the letter S. Tubules 11, 15 and 16 are short with enlarged, flattened, vesicular ends and are slightly curved. Tubules 1 to 16 inclusive are attached to the wall of the primary excretory duct. Tubules 17 to 34 inclusive are flattened vesicles and progressively decrease in size, the thirty-fourth being the smallest. This tubule is connected with the cranial end of the mesonephrogenic cord by a short, narrow pedicle, extending from its caudal surface. Tubules 8 and 9 are attached to the primary duct by means of a common stem, as are also tubules 11 and 12 and tubules 13 and 14. Communication with the lumen of the duct exists only in the first five tubules. The mesonephrogenic cord is 0.22 mm. long and is cylindrical in form, having a bluntly pointed caudal tip. It is somewhat irregularly constricted in several places and contains indifferently spaced, ragged, isolated lumina.

The right primary excretory duct is similar in construction to the left and is 3.05 mm. long. The cranial and caudal ends are slightly enlarged. There are irregular widenings along its entire length, but in general, from a point directly below the cranial enlargement to the end of its middle third section, the duct becomes progressively larger in diameter with lateral flattening, then progressively smaller, in a caudal direction, finally ending with a slight bulbous enlargement at the cloaca, to which it is attached. The wolffian bodies, measured from their centers, are 0.5 mm. apart at their cranial ends and 0.3 at their caudal ends. From both ventral and lateral views they are seen to curve in conformity with the curvature of the body of the embryo. In the caudal portion, when viewed ventrally, they curve with the tail to the right, and as the body of the embryo bends to the left in the region of their cranial ends, they too incline toward the left.

Alimentary Canal.—From a level above the lung buds to the tip of the tail gut, the alimentary canal, from the lateral aspect, appears as a curved tube, exhibiting on its ventral surface four out-pouchings, the rudimentary lung, stomach, liver and gall bladder, and also two tubular extensions, the vitelline and allantoic ducts. On its dorsal surface there is but one pouch—the developing dorsal pancreas. The curvature of the alimentary canal, with small deviations, parallels that of the body of the embryo. On each lateral surface of the canal may be seen a shallow groove, extending a short distance upward from the pulmo-esophageal notch and slightly differentiating the trachea from the esophagus. The lung bud is seen as a bilateral and slightly ventral diverticulum, extending from the caudal end of the trachea, its lateral wings tending to curve somewhat around the esophagus. No appreciable difference in the size of the lateral wings was noted. The lung bud, as also its lumen, is widest in its central portion, the lumen being continuous with that of the common tracheo-esophageal tube.

Caudal and dorsal to the lung buds, the alimentary canal presents a bulging of its ventral and, to a lesser extent, of its lateral walls, the right side more than the left. This represents the portion of the canal from which the stomach is developing. Its cranial and caudal ends appear as constrictions of the canal, the former being directly below the pulmo-tracheal junction, the latter directly above the dorsal pancreatic diverticulum. The greatest ventro-dorsal dimension of the gastric enlargement is found to be a short distance above its caudal constriction—the canal becoming gradually narrower toward the superior orifice of the stomach. The walls of the cranial and distal portions of the gastric enlargement are slightly thinner than those of the middle portion. The lumen of the stomach conforms to the external contour of the mucosa, as described above. The dorsal pancreas is differentiated from the duodenal section of the alimentary canal by a crescentic groove, which extends from the dorsal wall of the caudal end of the gastric enlargement, curving on the lateral wall of the canal in a ventral and caudal direction, and again downward and dorsad to form a duodeno-pancreatic notch.

On the right side the lower half of the groove is sharp and deep and the upper half shallow, while on the left side only the lower fourth of the groove is conspicuous, the remaining portion being quite shallow. On lateral view the dorsal pancreas appears as a dorsal and lateral oval out-pouching of the dorsal wall of the alimentary canal, with a slight tendency to angularity of outline. The dorsal wall of this pouch is slightly thinner than the lateral walls. The shape of the lumen in this portion of the alimentary canal conforms to its outer modeling, a constriction occurring along the line of the crescentic groove which forms the boundary between the duodenum and dorsal pancreas. The communication between the two is wide in a cranio-caudal direction and constricted laterally. The lumen in the lower tip of the dorsal pancreas forms a pocket behind an elevated thickening of the wall of the alimentary canal. This thickened portion forms the junction between the caudal wall of the dorsal pancreas and the wall of the duodenum. The hepatic diverticulum is attached to the ventral surface of the duodenal portion of the alimentary canal and consists of an oval sac from the upper portion of which the liver cords project. The wall of the lower portion of the sac, from which apparently the gall bladder is forming, is smooth and of fairly equal thickness. The hepatic portion of the diverticulum projects ventrally and cranially from the pedicle, broad cranio-caudally and somewhat flattened laterally. A slight notch separates the caudal tip of the sac from the alimentary canal. The lumen of the sac communicates with that of the duodenal canal through the short pedicle. The ventral pancreas apparently is not present in this specimen. Below the diverticulum, and caudalward to the center of the yolk-stalk attachment, the intestinal canal gradually becomes larger in diameter. From the liverbud to the cloaca the intestinal canal curves to the right and away from the midline of the embryo. The yolk-sac was removed before sections of the embryo were made. Its cavity communicated with the lumen of the alimentary canal through the vitelline duct. The shape of the duct is that of a flattened funnel, the mouth being attached broadly to the ventral wall of the intestinal canal. It curves ventrally and to the right of the canal. At the lower border of the yolk-stalk attachment the intestine is narrow, its size increasing progressively toward the cloaca, where it is again slightly constricted. Evidence of the development of the urorectal septum is seen in that portion of the cloaca directly caudal to the urorectal notch, where the cloacal walls, for a short distance, are apposed and in several spots seem to be fused, while here and there short delicate bridges of cytoplasm span the occasional narrow spaces between the walls. Depressions lying directly over the interior area of fusion, and corresponding to it in size, may be seen externally on both lateral surfaces of the cloaca. Extending from the depression, on each side, are two shallow grooves, the one directed caudalward to the middle portion of the tailgut, the other to the ventral wall of the alimentary canal at the junction between cloaca and tailgut, at which point the primary excretory duct is attached. The latter marks the junction between the flattened, funnel-shaped allantois and the cloaca. The tailgut groove on the right surface is sharper because of the acute curvature of the tailgut to the right, which causes a stretching of the left wall and consequent reduction in the depth of the groove.

The allantoic duct curves with the belly-stalk, in which it is imbedded, cranialward, ventralward, and to the left. It is an oval tube attached broadly to the ventral wall of the cloaca, from there on gradually becoming narrower to a point between the proximal and middle third of its length, widening and narrowing again in its middle portion and to the distal third, in which a uniform diameter is maintained, finally ending bluntly far out in the umbilical cord. The ventro-dorsally broad and laterally flattened tailgut terminates a short distance from the tip of the tail.

STAGE III.

(Embryo No. 1380, 5.5 mm. Sections 20μ, coronal. Figure 2.)

This embryo contains 38 somites. The wolffian bodies each lie between the lower level of the eighth and the lower level of the twenty-fifth somite, and their curvature parallels that of the embryo. Whereas in the 4-mm. embryo the caudal mesonephric tubules extend to a level opposite the middle of the cloaca, in this stage they end at some distance from and cranial to the cloaca. The cranial tubules in stage II extend to a level opposite the yolk-stalk attachment, while in this stage they extend to a level opposite the caudal end of the stomach. The length of the left mesonephros is 2.54 mm.—a difference of 0.41 mm. over that of the foregoing stage. The number of mesonephric tubules on this side has increased to 40. The first tubule is the smallest and its position is mesial and slightly dorsal to the primary excretory duct. It is made up of a short tubular and a small glomerular portion. The glomerular cavity is present, but apparently there is no lumen in the tubular portion. The tubule is attached to the cranial end of the mesonephric duct and appears to be undergoing dedifferentiation. The second unit is small, isolated from the mesonephric duct, and appears to be the atrophic remnant of the glomerular portion of a tubule, the body being invested on one side by a membrane, in appearance not unlike the capsule of Bowman. The third tubule is attached to the primary duct; it is S-shaped, the distal limb containing a small lumen. The glomerular portion and the secretory portion apparently have disappeared, the walls having the same appearance as those composing the tubulus collectivus and as seen in fully developed tubules. Tubules 4 to 27 inclusive are S-shaped and are attached to the primary duct. They are made up of three sections—collecting, secretory, and glomerular. In tubules 4, 5, 20 and 21 an ampulla is noted as an enlargement, situated between the two limbs of the S. Tubules 24 and 25 are connected with the primary excretory duct by a common stem. Tubules 28 to 31 inclusive are in the form of flattened, curved vesicles, connected with the duct by a short tubular portion. Tubules 32 and 33 are similar in form and are in contact with but not attached to the duct. Tubules 34 to 41 inclusive are flattened vesicles, each containing a small lumen, but are not attached to the duct. The tubules become progressively smaller caudalward. All of the tubules, except the first in the cranial end and the last four in the caudal region of the mesonephros, lie mesial to the primary excretory duct.

The mesonephric duct is about 3.1 mm. long. In general its diameter increased progressively in a caudal direction. Slight irregularities in size occur along its entire length, the lumen conforming to these variations. The duct shows some slight lateral flattening at the caudal end and on its dorsal surface is seen the metanephric rudiment. The latter, with the duct, is connected with the cloaca by a common tube 0.14 mm. in length. It consists of a small enlargement—the ureteral ampulla—situated at the point of junction between the mesonephric and common ducts, and surrounded by a somewhat conical cap of metanephrogenic tissue.

Alimentary Canal.—The alimentary canal, from a level slightly above the pulmo-tracheal notch to its caudal extremity, appears on lateral view as a tube roughly parallelling the body curvature. On its ventral surface and in the cranial portion appear two outgrowths, the rudimentary lung and liver, in the middle region the vitelline duct, and in the cloacal portion the allantoic duct. Caudal to the lung buds the stomach rudiment appears as a spindle-shaped enlargement of the canal. Directly beneath the caudal end of the stomach, and on the dorsal wall of the canal, is situated the dorsal pancreatic rudiment. The lung bud consists of two elongated vesicles extending lateralward and curving in a slightly caudal and dorsal direction from the trachea. The right vesicle is larger than the left. The distal end of each vesicle is slightly enlarged, as is also its lumen. A very definite constriction can now be noted between the tracheal and esophageal portions of the alimentary canal, but communication between their lumina still exists. That portion of the canal from which the stomach and dorsal pancreas are developing, when viewed ventrally, curves to the left of the midline of the embryo. The right lateral wall of the spindle-shaped stomach is somewhat flattened, the left bulging to form the rudimentary fundus. The cranial and caudal constrictions are much more pronounced than those in the alimentary canal of the preceding stage. Directly below the caudal constriction, growing from the dorsal wall and projecting slightly to the left of the canal, is situated the dorsal pancreatic rudiment. Its pedicle is narrower and the groove separating it from the duodenum is sharper than in the 4 mm. embryo. Its left wall is slightly flattened and somewhat thicker than the right. In this embryo there are no indications of the presence of a ventral pancreas. Below the caudal constriction of the stomach, and extending in a ventral direction, is the short ductus choledochus, connecting the hepatic diverticulum with the ventral wall of the duodenal portion of the alimentary canal. The liver is everywhere traversed by an intricate plexus of liver cords extending from the wall of the hepatic diverticulum. The ductus choledochus, with its lumen, is somewhat constricted at its point of attachment to the duodenal wall. It is a short, round tube projecting ventrally and to the right of the duodenum. Attached to its distal end are the hepatic and gall-bladder diverticula. The hepatic diverticulum extends in a cranio-ventral direction, its left wall bulging considerably. Its relatively wide lumen communicates freely with that of the ductus choledochus, but at its junction with the gall bladder the lumen is constricted, a corresponding constriction of the wall occurring at this point.

The gall-bladder appears as a laterally broad and cranio-caudally flattened vesicle, extending from the hepatic diverticulum in a ventral direction. Its lumen is narrow and conforms to the shape of its outer wall. Its free end is sharply pointed. From the promixal end of the stomach the alimentary canal is undulating. On ventral view it exhibits three distinct curves. The first curvature, bending toward the left of the midline of the embryo, includes that portion of the alimentary canal from which the stomach and dorsal pancreas are developing. Between the first and second curvatures lie the liver and gall-bladder. The second and third curvatures are to the right of the midline. On lateral view that portion of the canal between the middle of the second and the middle of the third curve bends ventralward to form the first loop of the intestine. To this portion the vitelline duct is attached.

The diameter of the vitelline duct at its junction with the intestine is small, widening progressively for the first quarter of its length, then becoming very narrow in its middle portion, finally widening again toward its junction with the yolk-sac. Traversing the cœlomic cavity of the umbilical cord with the omphalo-mesenteric blood-vessels, the duct swerves at first with the cord slightly to the left, and, at the line of amniotic reflection, abruptly to the right and out into the cœlomic cavity, where it communicates with the yolk-sac. Caudalward from the duct to the urorectal notch, the diameter of the intestine enlarges progressively with some ventro-dorsal flattening. The curvature of the hindgut, conforming to that of the body of the embryo, is not so acute as in the 4 mm. embryo. Some indication of the caudalward growth of the urorectal septum may be seen in the form of a groove on each lateral wall of the cloaca, extending from the urorectal notch well up toward the tailgut and dorsal to the common duct of the mesonephric and metanephric bodies. The cranial portion of the groove also serves as a junctional line between cloaca and allantois. The cloaca becomes gradually narrower toward the tailgut, which is short, maintaining a fairly even diameter throughout its length and ending a short distance from the tip of the tail. Beginning at the cloaca as a laterally flattened, funnel-shaped tube, the allantoic duct curves with the umbilical cord, in which it lies, ventralward and to the left of the body of the embryo and extends to the chorionic wall. Its caliber toward its distal end becomes very small, a slight enlargement occurring, however, near the end. The cloacal membrane forms a portion of the ventral wall of the cloaca and also of the allantoic duct. It is composed of ectoderm and entoderm, intimately fused, and represents the site of the future anal and urethral outlets.

Stage IV.

(Embryo No. 792, 8 mm. Sections 20μ, transverse. Figure 3.)

The curvature of the wolffian bodies no longer parallels that of the embryo, undulations being produced by means of pressure exerted upon them by the growth and downward shifting of adjacent abdominal viscera and by the increase in the size of their units. The left mesonephros is 2.4 mm. long (linear) and extends from the lower level of the second thoracic to the lower level of the fourth lumbar segment, and contains 38 tubules. On lateral view it curves dorsalward at the cranial end, then ventralward, and again dorsalward at its caudal end.

On ventral view it curves lateralward in its cranial half, then mesial, and again lateralward at its caudal end. The cranial ends of the wolffian bodies are farther apart than their caudal ends. In the cranial portion of the left mesonephros the tubules lie mesial, in the middle portion ventro-mesial, and in the caudal portion slightly dorso-mesial to the primary excretory duct. The tubules become progressively larger from the cranial end toward the middle and progressively smaller again toward the caudal end. With the exception of the first, they are all S-shaped. Tubules 19 and 20 are attached by a common stem to the duct; this is true also of 34 and 35, and 37 and 38 (fig. 12). The first and second tubules seem to be undergoing dedifferentiation. The first tubule is straight and projects, in a mesial and slightly dorsal direction, from the tip of the primary excretory duct. Mesial to it lies an isolated glomerular body. The other tubules are made up of a glomerular, a secretory, and a collecting portion. The collecting portion is narrower in diameter than the secretory portion. In some of the tubules ampullæ are to be seen between the secretory and collecting portions and also between the secretory and glomerular portions. In tubule 20 the glomerular cavity is very large. The tubules are considerably crowded. In some the S-curve is not so perfect as in others, having developed an extra kink in the collecting portion; furthermore, the limbs of the S are often bent toward each other. The primary excretory duct maintains a small, fairly uniform diameter as far as the middle of the mesonephros; from that point it becomes increasingly larger toward the most caudally placed tubules, where it again becomes much smaller and maintains the same diameter to the common duct, which connects it and the metanephric ureter to the wall of the urogenital sinus. It is somewhat flattened laterally. Its length (2.76 mm.) shows an increase of 0.22 over that of the 5.5 mm. embryo, and a comparison of the two embryos reveals a reduction of 2 in the number of tubules. The tubules show an increased growth in length and also in the size of the glomeruli in the caudal half of the mesonephros. The permanent kidney has grown in size, has shifted cranialward, and to some extent has assumed the bean-like shape of the adult organ. The upper pole is narrower than the lower, this difference in size corresponding to a similar one between the two ureteral branches, the upper branch being the smaller. The branches are represented by outpocketings of the blind ureteral end, which is imbedded in metanephrogenic tissue. Rotation of the transverse axis of the metanephros has begun. The ureter is now seen as a tube measuring 0.34 mm. in length and is attached to the dorso-lateral wall of the common duct. This duct has become much shortened. From both lateral and ventral views the ureter shows slight sigmoid curvature.

Alimentary Canal.—In this embryo the separation of the trachea from the esophagus is complete and the tracheal tube has developed a right and a left bronchus, from each of which four secondary pulmonary vesicles extend. The right bronchus is the longer. Both bronchi contain lumina continuous with that of the trachea, conforming in shape to the surface modeling. The stomach has increased in size and a rather shallow incisura angularis separates it from the flattened, funnel-shaped esophageal end. A shallow pyloric sulcus outlines the pyloric termination of the stomach. In a lateral view the stomach is seen to have shifted downward and from the vertical, as in the preceding stage, to an oblique position of about 45° to the transverse plane of the embryo. Viewed ventrally it curves to the left of the midline. In the preceding stage the distal end of the stomach is directly opposite the level of the first mesonephric tubule, while in this embryo it reaches to a point almost opposite the middle of the mesonephros. Due to the rotation of the stomach and the looping of the gut, the common bile duct now projects from the dorsal wall of the duodenum, and branching from it may be seen the ventral pancreas bud, the hepatic duct, and a short cystic duct, together with the much lengthened gall-gladder. From the dorso-mesial surface of the duodenum extends the dorsal pancreatic duct with the dorsal pancreas attached to it. The surfaces of the pancreatic rudiments are somewhat irregular. A single intestinal loop projects for a short distance into the cœlomic cavity of the umbilical cord and on its distal limb a cæcal pouch can be discerned. Beyond the pouch the large intestine continues caudalward to the urogenital sinus, to the dorsal wall of which it is attached. Communication between the rectum and urogenital sinus still exists. The tail of the embryo, which has become much reduced in length, contains a small vesicular remnant of the pinched-off tailgut. On the surface of the genital hillock are seen the intact urethral groove and membrane.

Stage V.

(Embryo No. 1909, 14.6 mm. Sections 20μ, coronal. Figure 4.)

The wolffian bodies extend from the level of the seventh thoracic to the level of the fourth lumbar segment. Their shape, from the lateral and ventral aspects, is somewhat sigmoid and their diameter is greater than in the preceding stage. The glomeruli have not grown much larger, but the tubules in the cranial half of the mesonephros have become slightly longer and those in the caudal half markedly longer, causing a considerable increase in the diameter of that portion of the mesonephros. All of the tubules come away from the primary excretory duct in a dorso-mesial direction. They are crowded against each other so as to form a fairly compact mass, which in the cranial portion lies dorso-mesial, coming eventually, toward the caudal end, to lie directly mesial to the primary duct. The cranial ends of the wolffian bodies are farther away from the midline of the embryo than are the caudal ends. The angle thus formed between their long axes is more acute than in the 8 mm. embryo. The length of the left mesonephros is the same as in the 8 mm. embryo (2.4 mm.), but the number of tubules has increased to 39. Cranial to the first definite tubule are seen isolated particles of tubular tissue which seem to be undergoing atresia. Whether these particles represent the remnants of completely or only partially developed tubules can not be definitely determined.

The first and second tubules are slightly S-shaped and possess rudimentary glomeruli. The diameter of their distal limbs is greater than that of the proximal limbs. The third and fourth tubules are not S-shaped but merely kinked, with slightly curved limbs, in which the glomerular portion is absent. Their distal limbs are enlarged. Examination of the sections reveals a condition of atresia. Tubules 5 to 11 inclusive are S-shaped, and, as in the four preceding units, the distal limbs possess a greater diameter than the proximal limbs. Tubules 11 to 39 inclusive, with the exception of tubule 28, are S-shaped and may readily be divided into a secretory and collecting portion. Some of them possess one to three ampullæ; in some instances one or two of these are situated between the collecting and secretory portions, that is, between the two limbs of the tubule, and represent the tubulus intermedius or tubulus ampullare, and another between the secretory and glomerular portion (tubules 18, 19, 23, 24). The twenty-eighth tubule consists of a glomerular portion and a short, poorly developed tubular portion. The left mesonephric duct is 2.72 mm. long. Its position in relation to the mesonephric mass is ventrolateral at its cranial tip to frankly lateral toward the caudal end. From there it courses in a medial and ventral direction, finally communicating with the urogenital sinus on its dorsal surface at a point directly mesial to the ureteral communication. As in the preceding stage, the diameter of the duct is greatest in the portion overlying the caudal half of the mesonephros. A funnel-shaped, laterally flattered enlargement may also be seen where the duct communicates with the urogenital sinus.

The alimentary canal of this specimen will be described in conjunction with that of stage VI.

STAGE VI.

(Embryo No. 966, 23 mm. Sections 40μ, coronal. Figure 5.)

The wolffian bodies in this specimen extend from the level of the second lumbar to the level of the fourth sacral body segment.

They are club-shaped structures with a slender cranial and a heavier caudal portion and, from the lateral aspect, practically parallel the curvature of the embryo. At their cranial ends they lie much farther apart than at their caudal ends. In the cranial portion of the left mesonephros are seen fragments of atretic tubules.

Tubules 1 to 5 inclusive are slightly S-shaped and consist of a glomerular, a secretory and a collecting portion. They appear to be undergoing a process of dedifferentiation. The lumina of these five tubules do not communicate with the lumen of the excretory duct. The secretory portion is larger in diameter than the collecting portion. Tubules 6 to 12 are S-shaped and are made up of the three portions, secretory, collecting and glomerular. Their lumina communicate with the lumen of the excretory duct. In these, as in the first five, the secretory portion has a greater diameter than the collecting portion. Tubules 13 to 20 inclusive extend from the primary excretory duct in a dorso-mesial direction. The collecting portion is long, the diameter being small. The secretory portion bends around to the mesial surface of the glomeruli, which surface it envelops, and its lumen communicates with that of the primary duct. In general, the secretory portion is larger in diameter than the collecting portion; however, in many units one finds several secondary enlargements of the tubule. Ampullæ are sometimes to be seen between the collecting and the secretory portion. Tubules 31 to 34 inclusive appear to be dedifferentiating. The secretory sections are not so large in diameter as in the tubules just described and do not exhibit the secondary enlargements. The twenty-ninth, thirty-first, and thirty-second are attached broadly to the primary duct. The thirty-fourth has a bifurcated collecting portion, the limbs of which are attached to the primary duct.

The Müllerian ducts have now made their appearance and extend from a level opposite the cranial extremity of the mesonephros to a point opposite the caudal end, where they blend with the neighboring epithelium of the primary excretory duct. Their position is cranio-lateral to the mesonephric ducts. They are flattened ventro-dorsally and increase progressively in lateral width toward the caudal ends. The cranial ends are slightly enlarged, but an abdominal ostium could not be made out. The left primary excretory duct is 3.0 mm. long and occupies a position lateral to the cranial half of the mesonephros, curving progressively in a medial and ventral direction toward the caudal end, and from this point continuing medianward and caudalward toward the urogenital sinus to the dorsal wall of which, close to the midline, it is attached. The diameter of the duct is widest in that part overlying the caudal end of the mesonephros and at its point of communication with the urogenital sinus. In the cranial portion of the mesonephros there may be seen what appear to be the atrophic remnants of the cranial end of the primary duct.

The kidneys have increased in size and have shifted from the pelvic to the abdominal cavity. In stage V the lower half of the kidney projects beyond the caudal extremity of the mesonephros, while in this stage it extends from the cranial tip to the middle of the mesonephros. The renal outer surface shows slight lobulation.

Alimentary Canal (Stages V and VI).—In comparing these stages with those previously described, the following changes may be noted: the shifting of the primary duct from the dorsolateral to the ventro-lateral position in the mesonephros, the relatively greater increase in growth of the stomach over that of the mesonephros, as well as the greater increase in the size of the liver, pancreas and kidney and in the length of the cystic duct, gall bladder and intestines. Comparing all stages, it is seen that from a vertical position in the first, second and third stages, with its descent into the abdominal cavity the stomach finally comes to lie (in stage VI) at an angle of 45° to the horizontal plane of the embryo. Its position relative to the mesonephros in the various stages is as follows: stages I and II, well above the cranial end of the mesonephros; stage III, its distal constriction opposite the level of the cranial tip; stage IV, its pyloric constriction well toward the level of the middle of the mesonephros; stage V, a trifle lower down; while in stage VI the body of the stomach extends from the level of the cranial end to the level of the caudal end of the left mesonephros and lies ventro-sinistral to it.

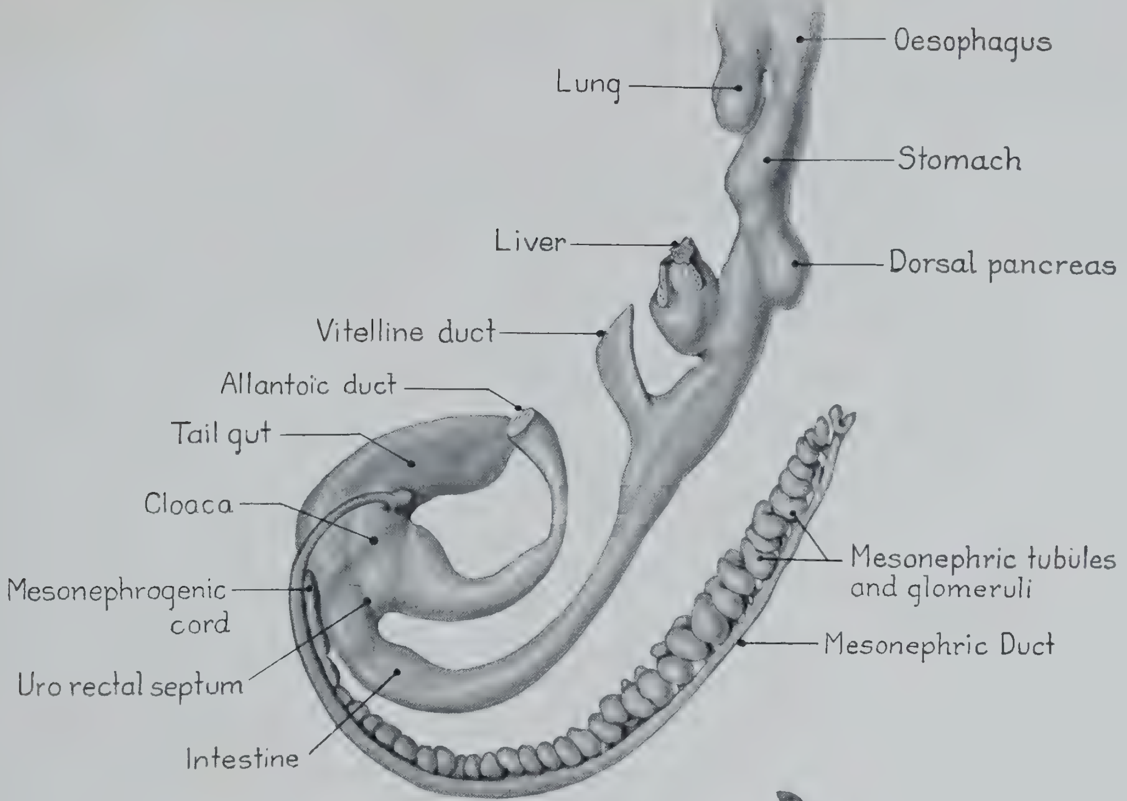
In stage V the fundus and corpus are well developed and the duodenum is separated from the stomach by a faint pyloric sulcus. This sulcus has become very marked in stage VI. In stage V the esophageal cone is clearly defined, while in stage VI the line between the esophagus and the body of the stomach is very shallow. In stage V the pylorus and duodenum together form a flexure, directed toward the right of the embryo, on which is seen the ventral pancreas fusing with the dorsal pancreas, the former to form the head, the latter the body of the adult gland. In stage VI the pyloric-duodenal flexure is directed toward the right and cranialward and the fusion of the two pancreatic rudiments is complete.

In stage V a single intestinal loop occupies the cœlomic cavity of the cord, from which the yolk-stalk has disappeared. In stage VI there are multiple convolutions of the small intestine, together with a part of the large intestine, in the cavity, forming the common umbilical hernia. On the most distal convolution a small cæcal pouch is seen, with a long vermiform appendix growing from it. In both of these stages the large intestine is shorter in length and smaller in diameter than the small intestine. In stage VI there is an ampullar enlargement of the large intestine a short distance from the anal outlet, which, together with the genital vestibule, has now been formed. The rectum has become separated from the urogenital sinus and a large genital protuberance is to be seen. From the lateral walls of the urogenital sinus the ureters extend for a short distance dorsally, then cranially to end in the renal pelvis.

GENERAL HISTOLOGICAL DESCRIPTION OF THE SIX STAGES.

(Figure 13, A to J inclusive.)

In stage I it is impossible to establish the presence of a continuous lumen in the primary excretory duct. This may be due to the tangential plane of sectioning in the cloacal region, but there is also the possibility that the lumen has not been completely formed, as it is not seen clearly in some regions where the section plane is favorable. In stage II the lumen is continuous and communicates with the cavity of the cloaca. Its wall is composed of a single layer of columnar cells arranged radially around the lumen and their oval nuclei tend to occupy the basal half of the cells. In later stages the cells become reduced in height. Stage II exhibits tubules developing from the mesonephrogenic cord and ranging from the simple vesicular and pear-shaped bodies, with cells radiating from the lumen, to the almost completely developed S-shaped tubules. The glomeruli in the latter are only partially developed. Mitotic figures are seen everywhere. The vesicular bodies have at first very small lumina; in the course of development these enlarge, the vesicles become elongated and grow in a lateral direction toward the primary duct to form the principal canal. Further, the canal develops into a pear-shaped structure and fuses with the primary duct. The wall of the neck of this tubule is composed of a single layer of columnar cells, while the wall of the body is made up of a double layer. The tubule then becomes lengthened and the formation of the S begins. A clump of blood-vessels from the aorta and cardinal vein grows into its distal end to form in part the glomerular portion. At this stage the cells composing the walls of the tubules show a similar staining reaction. Further elaboration of the S, increase in the size of the glomerular body, the formation of its capsule, the development of ampullæ, and the differentiation of the glomerulus, glomerular neck, secretory, intermediate (or ampullar), and collecting portions are seen in the final stages. The most striking difference between these secretory and collecting portions is the greater staining reaction of the cells of the latter, both nuclei and cytoplasm staining more deeply. In the cells of the two lamellæ of the glomerular capsule the nuclei stain more intensely than those in the secretory and intermediate portions. The cells covering the glomerulus are cuboidal in shape and have spherical nuclei. The outer lamella of the glomerular capsule is composed of pavement epithelium containing oval nuclei. In the glomerular neck the cells are either cuboidal or columnar, the former in tubules in which there is no ampulla in this region, the latter when the ampulla is present. The cells composing the wall of the secretory portion of the tubule are of the high columnar type with their nuclei in the basal half, while cuboidal cells with round nuclei compose the wall of the collecting portion. The transition from the cuboidal type of cell in the glomerular neck to the high epithelium in the secretory portion is gradual, while the line of demarcation between the latter and the collecting portions is fairly sharp. In none of the stages was it possible to establish the presence of nephrotoma. The cells composing the walls of the secretory portion of the tubules look very much like those seen in functioning glands. The ampullar enlargements are perhaps produced by pressure of fluid secretions, for in the tubules undergoing atresia the ampullæ have disappeared. It is on the basis of these observations that the author ventures the opinion that the mesonephros has a functional property.



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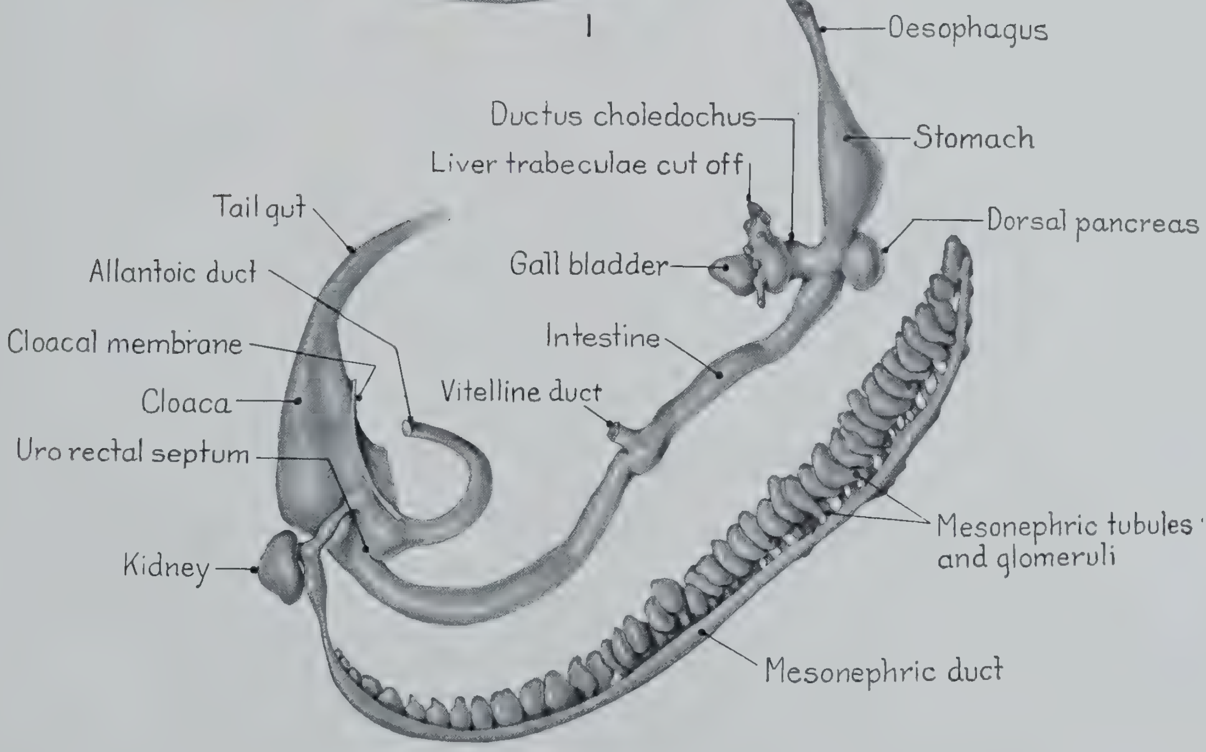

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FIG. 1. Geometric projection from a reconstruction model of the structural details of the mesonephros, together with the alimentary canal, of a 4-mm. human embryo (Carnegie Collection, No. 836), stage II. ×50.

FIG. 2. Drawing, obtained in a similar manner, of the mesonephros, metanephros, and alimentary canal of a 5.5-mm. human embryo (C. C. No. 1380), stage III, to show their structure and relation to one another. ×50.

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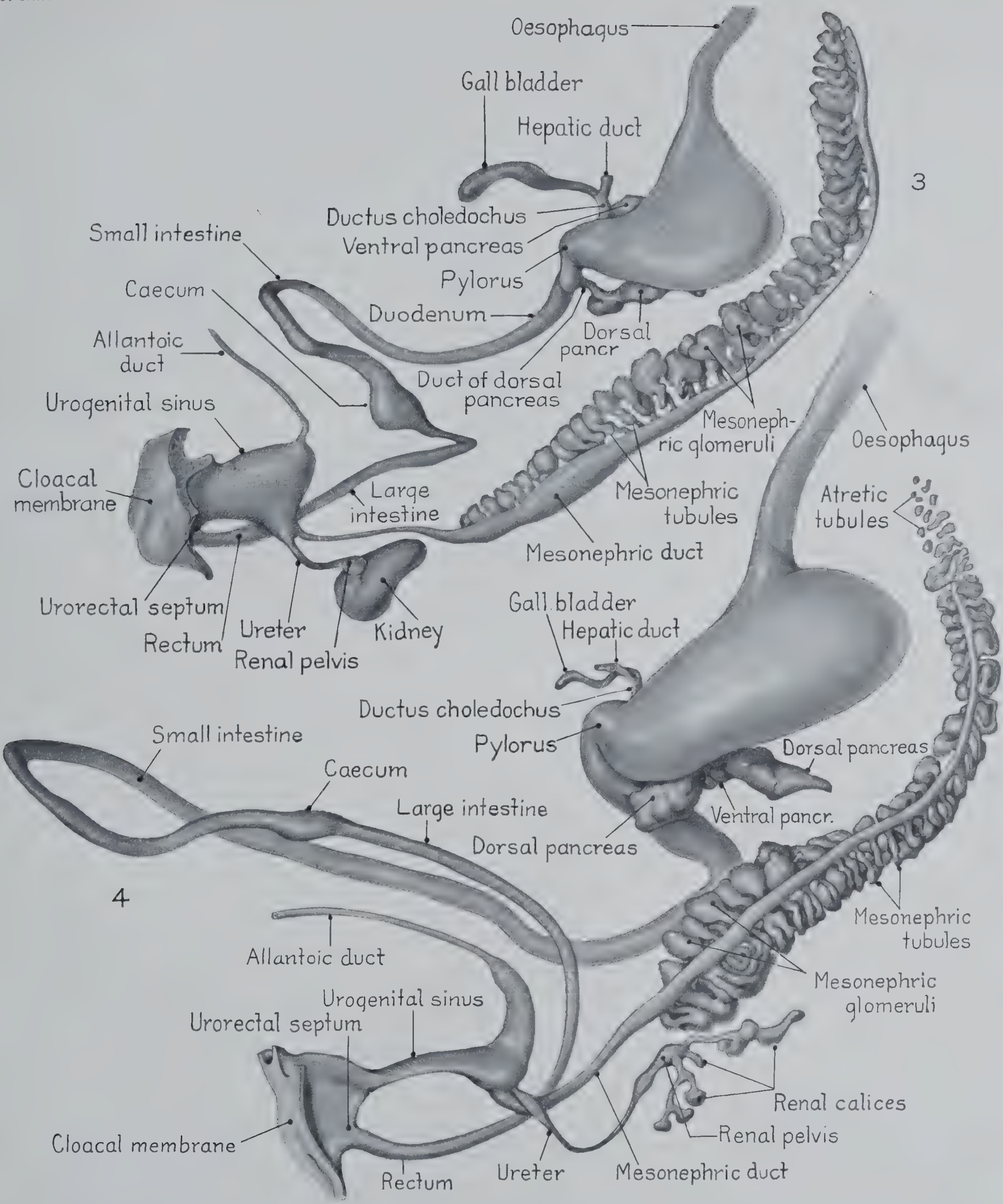

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FIGS. 3 and 4. Geometric projections from models of the mesonephric tubules, metanephros, and alimentary tract of human embryos 8 mm. and 14.6 mm. long respectively (C. C. Nos. 792 and 1909, stages IV and V), showing their further development and their relation to one another. ×50.

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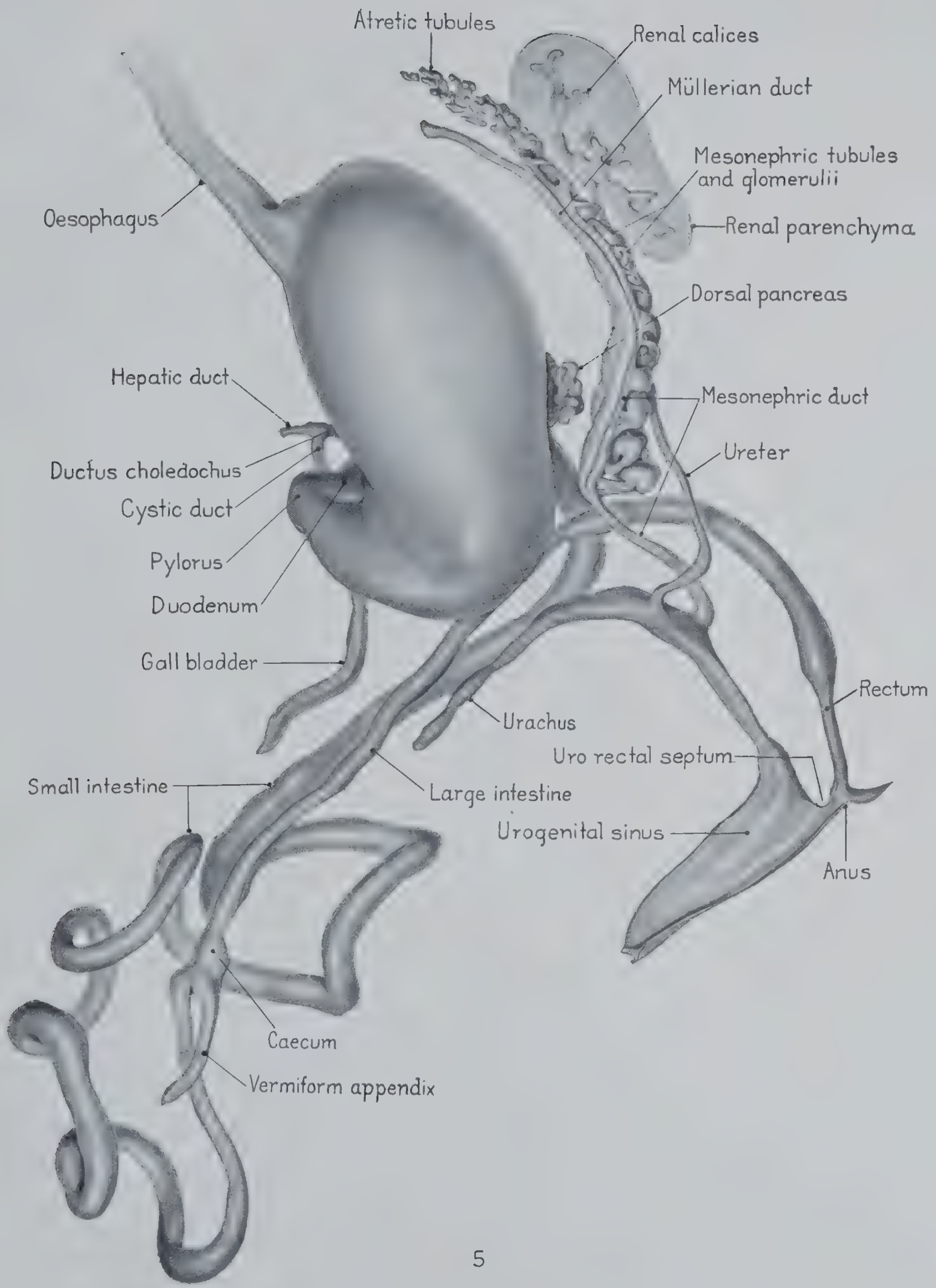

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FIG. 5. Geometric projection from model of the tubular units in the mesonephros of a 23-mm. human embryo (C. C. No. 966, stage VI), showing their structure and relation to the metanephros and intestinal tract. ×30.

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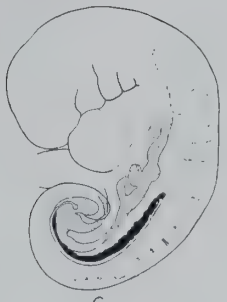

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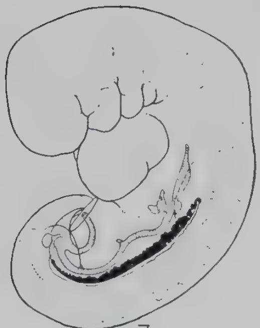

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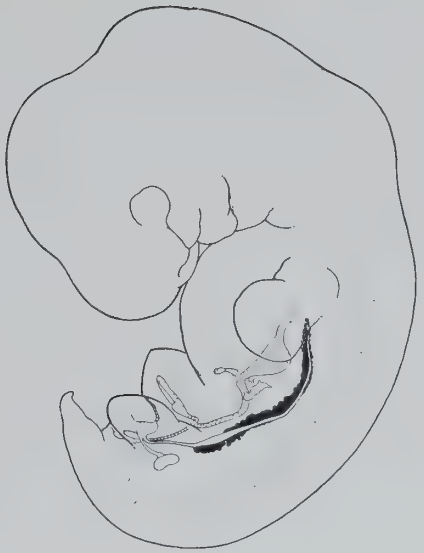

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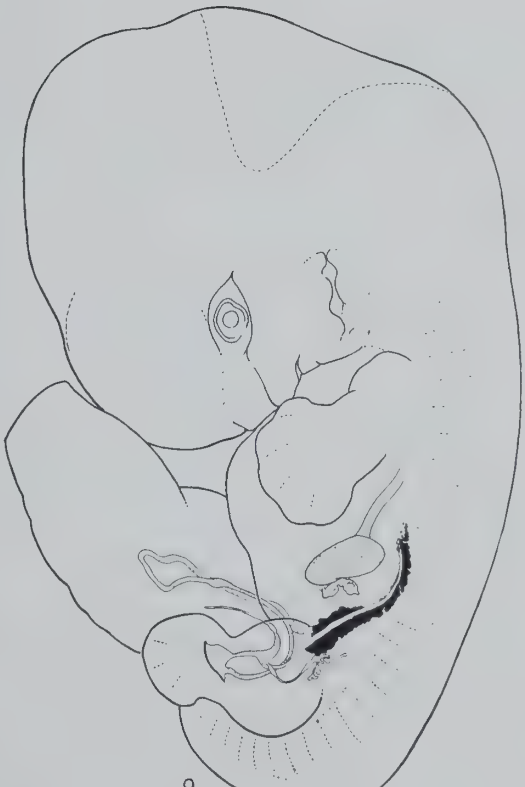

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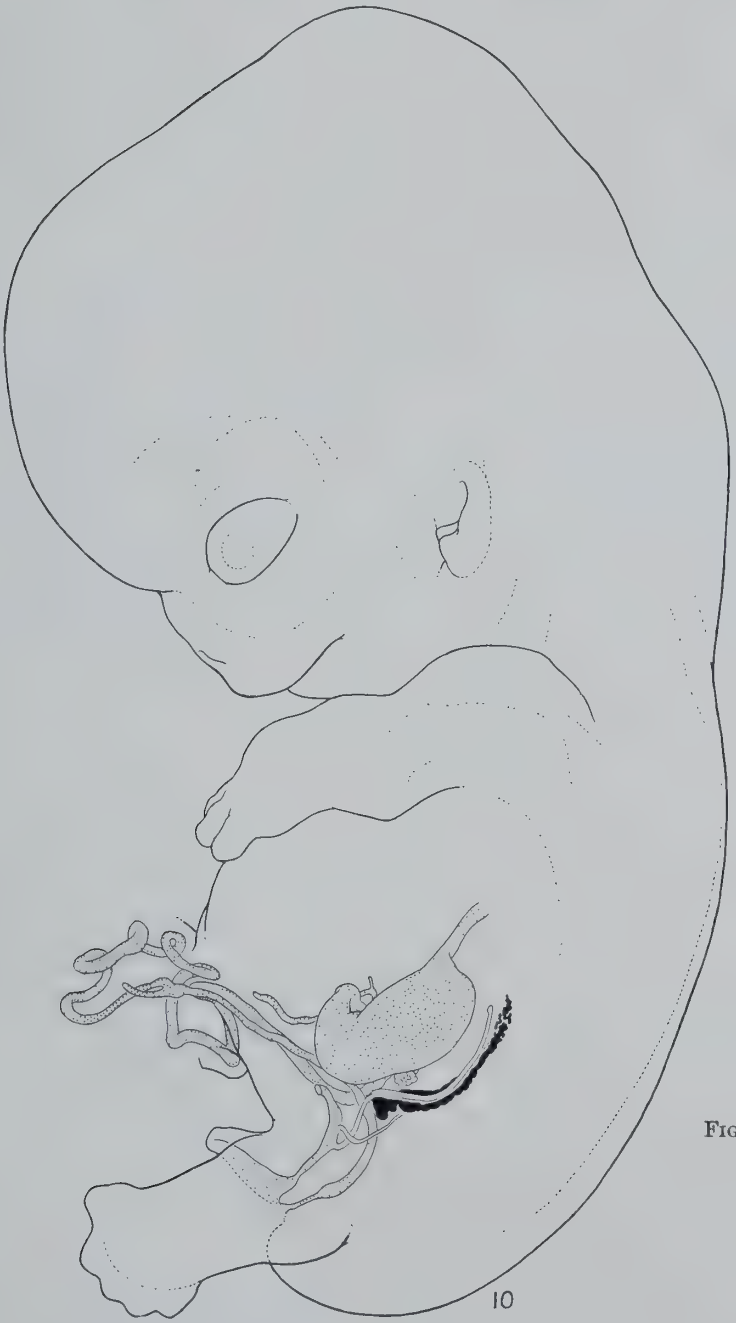

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FIGS. 6 to 10. Semi-schematic drawings, based on geometric projections, of human embryos of 4, 5.5, 8, 14.6 and 23 mm. respectively, to show the location of the mesonephros and alimentary canal in the body of the embryo at different stages of development, as well as their relative rates of growth.

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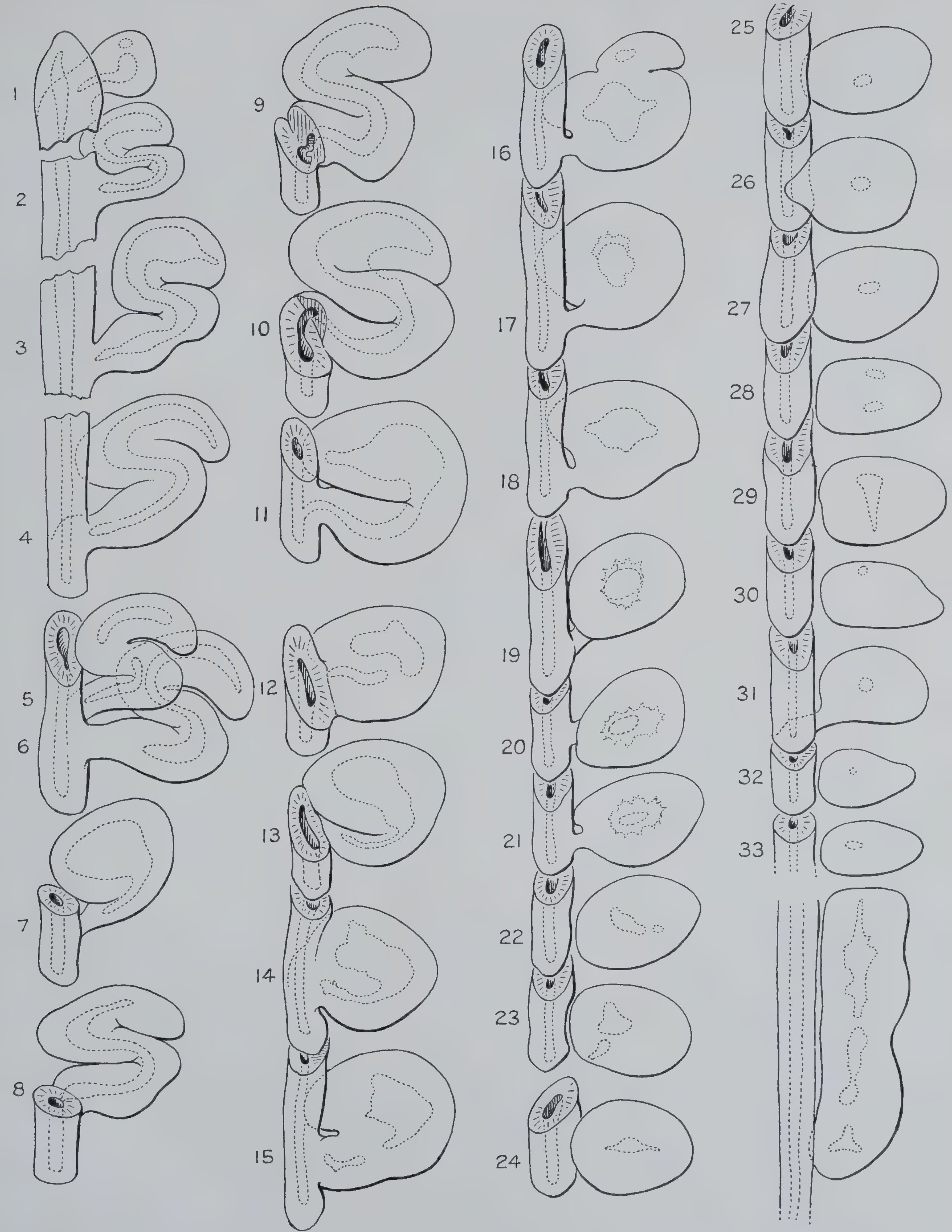

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FIG. 11. Semi-schematic drawings based on camera-lucida projections of sections of the mesonephric tubules of a 4-mm. human embryo, showing number, shape, and size of the tubules and their relation to the primary excretory duct (C. C. No. 836, stage II).

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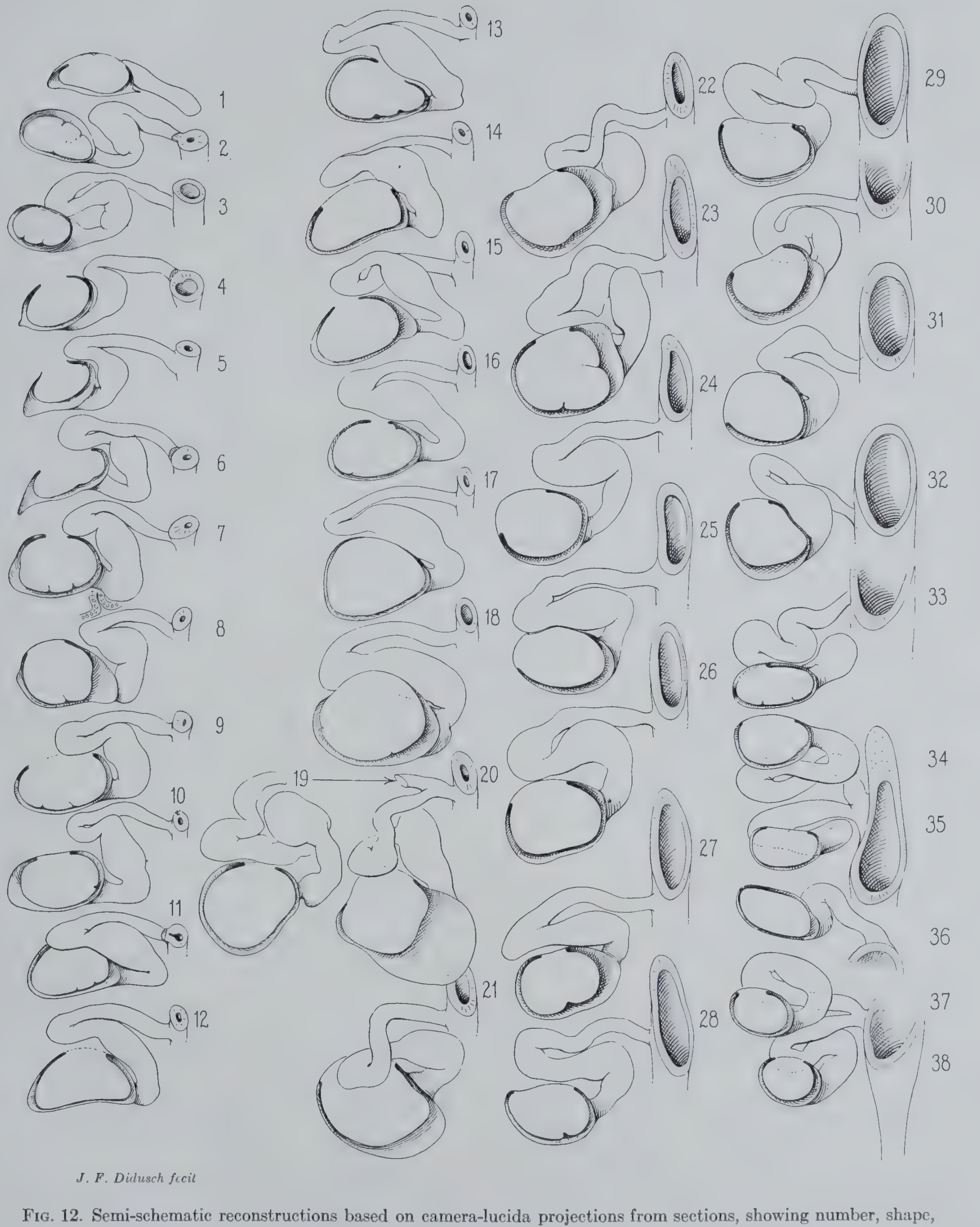

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FIG. 12. Semi-schematic reconstructions based on camera-lucida projections from sections, showing number, shape, and size of the mesonephric tubules and their connection with the primary excretory duct, in an 8-mm. human embryo (C. C. No. 792, stage IV).

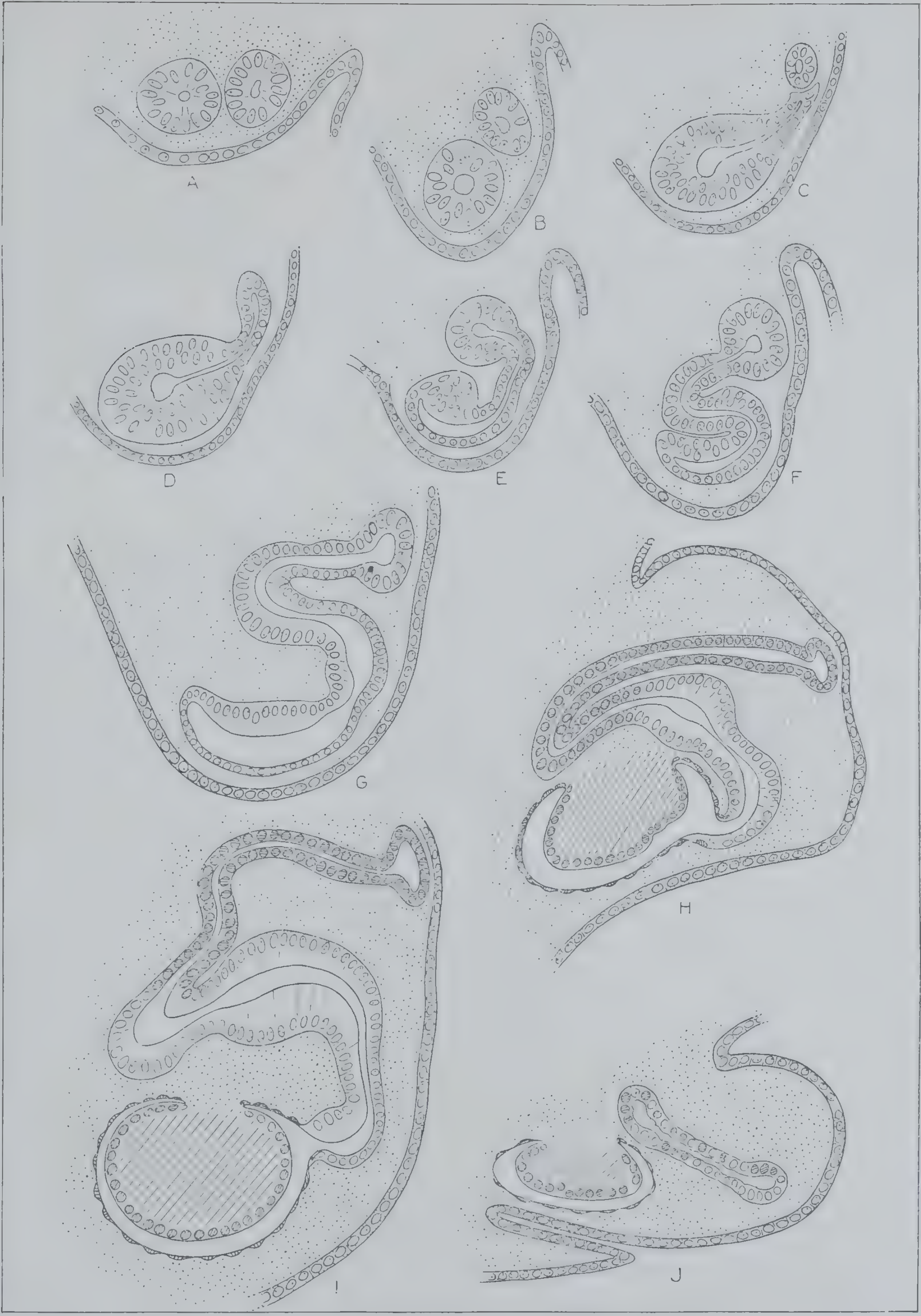

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FIG. 13, *a* to *j*. Semi-schematic diagrams based on camera-lucida outlines made from sections, to show the development of the S-shaped mesonephric tubular unit from the simple mesonephrogenic vesicle and its dedifferentiation. It was necessary in some of these stages to combine the outlines of several sections, since it was not possible to obtain a complete outline of the more complicated forms in a single section. The outlines were selected from the mesonephroi of the six embryos described in the text, with a view to showing successive degrees of development possible in a single tubule.

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CONTRIBUTIONS TO EMBRYOLOGY, NO. 94

LENS ECTODERM AND OPTIC VESICLES IN ALLANTOIS GRAFTS.

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With two plates.

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LENS ECTODERM AND OPTIC VESICLES IN ALLANTOIS GRAFTS.

I. INTRODUCTION.

The study of growth and differentiation of isolated chick primordia and parts of blastoderms grafted into the allantois discloses a great diversity in the behavior of the transplanted tissues. Murphy's ingenious method of grafting tissues of tumor and adult spleen into the allantois seemed to me to be particularly well adapted for use in the study of growth and development of isolated embryonic structures. When transplanted into the allantois, the various primordia seem to differ greatly as to their development, and only a thorough study of every structure in its different stages of development can determine both the potencies and limitations of a group of grafted cells and tissues and the degree of their dependence upon local factors for their differentiation.

Morphological differentiation, as we see it effected in a normal structure, seems to be in many cases the result of factors inherent in a group of embryonic cells, regardless of whether we connect them with the constituents of the nucleus or with the constituents of the cytoplasm. In typical development differentiation leads, with law-like immutability, to normal structures. But the sum total of conditions which we term "typical development" is of such intricate complexity that only experiment, supported by a careful analysis of deviations from normal development observed in nature (monstrosities) can help us to determine the respective significance of each series of factors.

The eye-lens, a highly specialized structure, develops in a normal embryo at the expense of a definite area of ectoderm. It is futile, however, on the basis of observation alone, to attempt to decide as to the nature of the factors determining the series of changes in the ectodermal cells which lead to their differentiation into the lens. Observations on the typical development of the lens can neither confirm nor disprove any of the following possibilities.

(1) That differentiation of the lens may be determined by the specific structure of the cells in the definite area of ectoderm. In this case nothing but a lens can develop from the ectodermal cells of this definite area. Wherever transplanted they must give a lens, provided conditions are favorable to their existence.

(2) That differentiation of the lens, in addition to the special structure of its primordium, may demand for its completion special factors, such as contact with the eye-vesicle. In the absence of these factors no lens can develop. If transplanted into another part of the organism without the eye-vesicle, the lens primordium will fail to develop into a lens. Neither will it differentiate in its normal position if the eye-vesicle has been destroyed or displaced. On the other hand, wherever transplanted together with the eye-vesicle, it will transform into a lens, provided no other factors are involved in this differentiation. Another part of the ectoderm, however, even though contact between it and the eye-vesicle be secured, will not transform into a lens.

(3) Finally, that the potency for differentiation of a lens may belong not alone to the definite area of ectodermal cells which, under normal conditions, undergo this transformation; it may prove to be a general property of all embryonic ectoderm. In this case, any part of the ectoderm, if subjected to definite conditions, may undergo the same transformation as that exhibited under normal conditions by a definite area only.

W. H. Lewis (1904) and Le Cron (1907) have interpreted the differentiation of the lens as a partial expression of a general property of the ectoderm. The results of their experiments on amphibians demonstrate not only the existence of a remarkable interaction between groups of tissues while they are developing into an organ, but also seem to prove that, in the typical development of the lens in amphibians, the factor determining the transformation of the ectodermal cells into a lens is contact between the eye-vesicle and the ectoderm. Since such contact with any part of the ectoderm calls forth a lens differentiation, it would seem that in amphibians the potency to produce a lens must be regarded as a general property of the ectoderm.

However interesting these results may seem, their significance is limited by the fact that they apply only to a definite class of animals. This class is well known to possess a high degree of regenerative capacity, which is lacking in higher animals. Regenerative capacity is often based upon retention by the organism of a stock of highly polyvalent cells whose presence in higher animals can no longer be observed.

In our efforts to analyze the respective potencies of various tissues in higher animals, and to determine their gradual ontogenetic and possibly phylogenetic limitations, we have to be very careful in ascribing properties characteristic of tissues in a class of lower animals to analogous tissues of higher animals.

II. MATERIAL.

The method of transplanting isolated chick primordial structures and parts of blastoderms into the allantois of another developing chick embryo has been used by me since 1916, and a large amount of material bearing upon the differentiation of the lens has been gradually amassed. An opportunity to add an analysis of the development of the lens under very unusual conditions in a human embryo has been given to me by Dr. G. L. Streeter, who has placed at my disposal a stunted human embryo, cut into serial sections and reconstructed by Mr. O. O. Heard.

Grafting into the allantois seems to differ considerably from transplanting rudimentary structures under the skin in other regions of the same organism, as has been done by Lewis in amphibians. Under normal conditions, the chorionic ectoderm covering the allantois does not undergo cornification, as does the ectoderm of other parts of the embryo skin. Nevertheless, as described by me in 1918, it is capable of cornification and often undergoes such changes in the area over and in proximity to the graft. A more fundamental difference in the two methods consists in the vascularization of the graft during its implantation. In the allantois the tissue grafted is deposited on a rich vascular net which, by its immediate ingrowth, easily secures a further growth of the tissue. On the other hand, the rapid and abundant ingrowth of vessels accompanied by mesenchyme often effects a subdivision of a single structure into a number of smaller parts. Moreover, unusual connections between vessels and cavities of other organs are frequently established in an allantoic graft, such as cavities of the brain and spinal cord, of the esophagus, and of the small tubules of various organs.

Series of experiments were planned in order to determine the respective value of factors inherent in the group of ectodermal lens-forming cells and of factors supplied by outside conditions. As contact with the optic evagination, especially, had been claimed to be an outside factor determining the special changes observed in the ectoderm during lens development, the experiments were planned with a view to ascertaining the relationship existing between the optic vesicle and the lens.

(1) The first series of experiments was undertaken to determine whether or not the group of ectodermal cells which normally develop into a lens (called henceforth in this paper *lens ectoderm*) effect this transformation by virtue of their specific structure alone. Differentiation of these cells into a lens in a region of the organism in which none of the habitual conditions are present would constitute good evidence that differentiation is not dependent upon local conditions. The special nature of the lens ectoderm would become evident only in case it could be shown that no other part of the ectoderm will produce a lens under the same conditions. A great number of experiments were arranged in which the area of the ectoderm over the optic evagination had been cut out, cleared of every bit of adjacent tissue and transplanted into the allantois. These experiments are described in section III. Failure of the transplanted lens ectoderm to develop into a typical lens, as established in this series of experiments, though important as corroborative evidence, can not, however, be interpreted as final proof of the impotency of the isolated and transplanted ectodermal cells to produce a lens away from their habitual environment. Injury during isolation of the thin membrane and inability to supply the grafted ectodermal membrane with normal invagination conditions might have inhibited or modified their further normal development.

(2) Another series of experiments was planned with the view of determining whether or not some outside factors are necessary to bring about the differentiation of the lens ectoderm into a lens; in other words, whether the optic evagination, claimed by Lewis to be the determining factor in this differentiation, is necessary for the development of the lens ectoderm into a lens. If so, the removal of the optic vesicle should be accompanied by failure of the lens to develop.

To remove the optic evagination in a chick embryo at an early stage of its development without injury to the ectodermal layer covering it is very difficult. There is no chance of success if it is done in the manner practised by Lewis on the tadpoles, i. e., by lifting a skin flap, cutting out the eye-vesicle, and returning the ectoderm to its original place. It is easier to gain access to the optic vesicle from the entoderm side, but even in this case an injury to the whole cephalic region of the blastoderm can not be avoided, and therefore the value of this set of experiments is rendered uncertain in a discussion of the potencies of the lens ectoderm. No analogy can be drawn between Lewis' extirpation of the optic vesicle in amphibians and the experiments on chick blastoderms, in which there were removed from the area of the lens ectoderm tissues including the optic vesicle, the entoderm, and the adjacent mesoderm, with subsequent transplantation of the cephalic part of the blastoderm into the allantois. I was fortunate, however, in obtaining a large number of grafts of the head process region of the blastoderms in which one or both optic vesicles failed to develop. Without being able to account with certainty for this rather unexpected event, I think these grafts may well be used in an analysis of the reciprocal relationship of lens ectoderm and optic vesicle. These grafts are described in section IV(a).

A number of transplants of both lens ectoderm and optic vesicle were made in order to ascertain whether or not the optic vesicle will determine lens differentiation in lens ectoderm which fails to develop if grafted alone. The results of these experiments are described in section IV(b).

3. No special experiments were needed to determine whether or not the optic evagination has the power to call forth in any part of the ectoderm the special changes resulting in lens differentiation. In grafts of either the whole blastoderm or the cephalic part, displacements of various structures occur. Optic vesicles in the form of long, convoluted tubes are seen to grow out from the brain and frequently are pushed aside from their normal position, either by vessels or by the ingrowing mesenchyme. Their distal ends may be found in almost any region, as for example, under the ectoderm of the heart or liver. In a number of such grafts the optic vesicles have undoubtedly effected a contact with a region of the ectoderm situated at a considerable distance from the lens ectoderm. These grafts are described in section V.

Finally, the eye region of a human embryo is described in section VI. This embryo, even though not furnishing further evidence of the potency of the eye-vesicle to produce a lens from any part of the ectoderm, affords great interest in demonstrating a considerable independence of development on the part of the organs, and at the same time a close interrelationship between groups of tissues in building up an organ.

III. LENS ECTODERM IN TRANSPLANTS.

The lens ectoderm is an integral part of the general ectodermal surface of the embryo. Its initial changes during development into a lens are observed at the time the optic vesicle establishes an intimate contact with it. According to the table given by Lillie (1908), the lens is indicated by an ectodermal thickening at the stage of 21 somites. My own observations tend to place these processes at an earlier period—that of 7 to 10 somites, at a time when the optic vesicle, not yet constricted at its base, forms a distinct evagination. The specification of the lens primordium would seem to take place at a very early period, and in using the transplantation method to test the potencies of the lens ectoderm, care should be taken to work with very early developmental stages.

Thin, almost transparent fragments of ectodermal membrane, dissected out in Ringer's solution from the eye region of the blastoderm, were quickly transplanted into the allantois of another chick embryo of 7 to 9 days' incubation. In a great number of cases the thin membrane could not be found when the egg was opened again after a few days of further incubation. A practice was therefore adopted to section serially the whole area of the allantois situated under the opening made through the egg shell through which the graft was deposited. Thus a number of cases have been secured in which the allantois showed upon microscopical examination changes which are not exhibited by this membrane without grafts.

As may be remembered, a rich vascular net is seen to develop, from the ninth day, within and over the ectoderm of the chorion, which at that time is fused with the allantois proper. Placed in direct contact with this vascular net, the fragments of the grafted ectodermal membrane are promptly overgrown by mesenchyme and numerous capillaries. The ectodermal cells of the chorion begin themselves to proliferate in response to the contact with the tissue brought in, and soon form a continuous covering over the graft. The grafted ectodermal membrane is thus found surrounded by mesenchyme and conditions are thereby obtained which are similar to those under which the lens is normally formed. The invagination of the grafted ectoderm is effected by overgrowth of the chorionic ectoderm. Of course no eye-vesicle is present.

The grafted ectoderm not only continues to live under these conditions, but proliferates and undergoes a series of changes. Mitoses are frequently seen. The changes which the lens ectoderm undergoes within the allantois, similar in nature in all grafts, differ in results according to whether or not the grafted material has been fragmented into a number of parts by ingrowing vessels.

If the thin, flat ectodermal membrane is respected by the adjacent vessels, its cells exhibit an unmistakable tendency to rearrange themselves in the form of a more compact structure. A number of them are seen to elongate, to become fusiform, and to arrange themselves in a more or less regularly concentric way around a point which may be a few mesenchymal or blood-cells. Those inclined to believe the lens primordium to be specific may readily identify this structure as an imperfect lens. True, no trace of a cavity can be recognized in it, therefore, no typical epithelial layers are present, neither the anterior membrane, which normally consists of flat cells, nor the posterior one, formed by the lens fibers. This irregularity in the development may, however, depend upon the different way in which the invagination of the grafted ectodermal membrane is effected in the transplant, as compared to the way it is completed under normal conditions.

Without denying a certain similarity between such structures and an eye lens, I would like to point out the fact that structures identical with those obtained in grafts of the lens ectoderm in the allantois are frequently observed under natural and experimental conditions. The Hassal corpuscles in the thymus, believed to represent the epithelial remnants of the entodermal rudiment of the organ, and the epithelial pearls often observed in carcinoma are strikingly similar to the epithelial structure described above. Moreover, as represented in one of my papers (1918, fig. 1), groups of ectodermal cells of the chorion may become isolated while a grafted tissue is growing in the allantois, be pushed deeper into the allantois membrane, and there assume the form of epithelial pearls—in the case cited, rather small and numerous. Again, in the experiments on tadpoles, while piercing the ectoderm with a thin glass pipette in order to introduce different kinds of particulate protein into their fins, groups of epithelial skin cells were occasionally pushed into the mesenchyme and seen to form there epithelial pearls.

Frequently, in the case of grafts of lens ectoderm, a larger number of pearl-like structures (up to five or six) are formed, that is, when the grafted ectodermal membrane has been split into smaller parts by ingrowing vessels.

Should the property of building a spheroidal structure, so generally exhibited by the surface epithelium when dislodged and inserted within the loose mesenchyme, still be attributed to the lens ectoderm, as its special endowment? In order to find out to what extent this capacity belongs to the surface ectoderm in general, a great many grafts of parts of the surface ectoderm were made at different stages. If retained within the allantois and overgrown by its mesenchyme, they invariably formed pearls.

Thus, the results of the first set of experiments have shown the property to form spheroidal structures, with concentric arrangement of elongated cells, to be characteristic of the lens ectoderm, as well as of the surface ectoderm in general. The structures thus obtained are, however, not real lenses. Should the failure of the lens ectoderm to develop into a typical lens within the allantois be regarded as an evidence of incapacity of self-differentiation in general? May not this failure possibly be attributed to inhibition, brought about by conditions in the experiment? Should the factor claimed by Lewis to be the determining one in lens differentiation (contact of the rudiment with the optic vesicle), if retained in the experiment, supply the missing condition for development of the lens ectoderm into a real lens? This part of the problem is discussed in section IV.

IV. IS THE OPTIC VESICLE A FACTOR IN LENS DIFFERENTIATION?

A. ELIMINATION OF OPTIC VESICLE.

If the lens ectoderm, isolated from the optic vesicle and grafted into the allantois, does not produce a lens because of a definite, still undetermined condition connected with the absence of the optic vesicle, it should also fail to form a lens in its normal position if the optic vesicle be removed. A blastoderm from which one of the optic vesicles had been eliminated grew within the allantois in a way not greatly different from that in which an intact blastoderm would grow in a graft. In such blastoderms, however, two lenses could never be found. Even in those in which the optic vesicle had not been completely removed and a rudimentary cup had been formed in the shape of a short, pigmented stalk without connection with the ectoderm, neither a lens nor pearls could be found in this region.

Even though this observation be only a negative result, the regularity of its occurrence points decisively to the existence in normal development of a more intimate connection between the two primordia than mere synchronism. Lenses do not develop within the allantois except conjointly with the optic vesicle and in intimate contact with it. A study of grafts of blastoderms or of their cephalic parts brings forward new evidence for the necessity of an association between the two primordia for lens differentiation.

Only those well acquainted with the results obtained by the method of transplanting whole or parts of chick blastoderms into an allantois will realize the difficulty of retaining the parts of the blastoderm and the various primordia in their respective positions. The thin blastodermal membrane folds easily, because the tension which normally exists within it when it is spread over the yolk-sac is not present in the graft. The ramifications of the allantoic vessels that develop in a grafted blastoderm frequently bring about dislocation of the organs. Finally, the allantoic membrane, which by itself is a loose tissue and is often found in the graft, furnishes a rather uniform, soft medium for the greatly varying conditions of pressure and tension which prevail between structures of different density in normal development.

In regard to the primordia under discussion, the conditions in the allantois greatly modify the development of the optic evagination. The latter develops in a more or less regular way only where the rudiment itself or the part of the blastoderm which contains it is grafted at a stage when the invagination of the optic cup has well started. If grafted in earlier stages, the optic evagination takes the form of a long, narrow tube, the lumen of which is scarcely more than a virtual one. The tube grows rapidly; it folds and winds, and in sections simulates a glandular structure. It usually merges with the brain tissue at a sharp point at which it communicates with the large brain cavity by a narrow orifice. Occasionally a dilatation may appear at some point of the tube (fig. 2), but this is a rare occurrence. The cavity of such dilatation, as well as the lumen of the whole tube, is in direct continuity with the brain cavity. This malformation of the optic vesicle regularly occurs in a graft of a young rudiment and should be considered as due to the lack, in the allantois, of conditions which, in a normal development, regulate the molding of organs into a definite form. A group of embryonic cells transplanted into the allantois proliferate and often differentiate in the same way as in typical development; but, growing under conditions which mechanically are different from the normal ones, they assume a different arrangement, resulting in a difference in the final form of the organ. In the case of the optic vesicle, it grows in the loose mesenchyme of the allantois as any other epithelial invagination would progress in loose embryonic mesenchymal tissue—in the form of a glandular structure.

A most peculiar feature of an optic vesicle developed in a graft from a young specimen is that all of its cells are pigmented—those forming the convoluted part of the tube and those of the occasional dilatations, as well as those which directly apply to the lens or to its rudiment. The pigmented cells terminate abruptly in the region where the optic vesicle merges with the brain tissue. As seen in figures 1, 3, 5, parts of the walls may appear thickened and considerably less pigmented, but upon closer microscopical examination granules of pigment are revealed in every one of the cells, though in some of them in smaller quantity.

It will not be possible, in this paper, to discuss the failure of the retinal layer to develop in a typical manner.

Displacement of the optic vesicles, or rather of their distal ends, is a frequent occurrence. It regularly takes place in a graft of the cephalic part of the blastoderm which, at the stage of 5 to 8 somites, has been severed from the caudal part close behind the optic vesicles and contains the primordium of the forebrain with the optic vesicles. The medullary tube closes readily at its sectioned end and this brings the two optic vesicles close together. Four to six days after transplantation, a graft of this kind shows a luxuriant growth of the forebrain, a description of which must be omitted in this paper. The optic vesicles, as seen in figure 5, appear in the form of a mass of tubular, coiled structures, and are often separated from each other by rather inconsiderable strands of mesenchyme.

Modification in form and displacement are not the only changes incurred by the eye primordium in a graft of the cephalic part of a blastoderm. In a number of grafts no optic vesicle at all was found after 3 to 7 days' growth. An eye, as changed as it may appear in a graft, can be readily recognized by the presence of pigmented granules within the cells. They appear early, develop in all the cells of the optic evagination, and make the identification of the organ easy, whether the latter has developed well or consists of only a rudimentary stalk. Absence of pigmented epithelial structure in a graft may be taken as reliable evidence of failure of the organ to develop. A careful study of grafts in which either one or both of the optic vesicles have failed to develop has revealed also the failure of the lens of the corresponding side to develop.

In grafts of blastoderms in which the optic vesicle has not developed at all, or, owing to its peculiar alteration in form, has grown away from its normal location, the lens ectoderm can not be recognized as such. It is evident, however, that the lens ectoderm can not follow the optic vesicle in its displacement to the full extent. The optic vesicle, growing in the form of a long and narrow tube, slips between the various organs, and in one of my blastoderm grafts it appears between the liver and the heart. The lens ectoderm, being part of a continuous membrane, can not possibly undergo a displacement as extensive as does the optic vesicle. If it should possess a potency to develop a lens without close association with the optic vesicle, there should be found lenses developed under the ectoderm in the region of the brain. It is significant, however, that in blastoderms in which the optic vesicle has grown away from its normal location no lens has been seen to develop other than in closest connection with the optic vesicle. Whether transplanted alone (first series of experiments) or left in its normal place but disconnected from the optic vesicle (second series of experiments), the lens ectoderm seems to be incapable of differentiating into a lens.

B. LENS ECTODERM AND OPTIC VESICLE IN TRANSPLANTS.

The necessity for an association between the lens ectoderm and the optic evagination is particularly well demonstrated by a series of grafts of lens ectoderm with attached optic vesicles. It has been found more practicable to graft one eye rudiment at a time, the contact between lens ectoderm and optic vesicle being more easily secured in this case. Figures 1 and 2 represent two typical cases of such grafts, figure 1 after 3 days, figure 2 after 5 days' growth in the allantois. In both cases beautiful lenses have developed in intimate connection with the optic vesicles. A detailed description may be omitted in this case in view of the clearness of the pictures.

There seems to remain no doubt as to the significance of the optic cup in lens differentiation. Transplanted alone into another part of the organism, the lens ectoderm fails to develop into a real lens, but, as any other part of the surface ectoderm, is transformed into pearls if overgrown by mesenchyme. Left in its normal position but disconnected from the optic vesicle, it remains an integral part of the surface ectoderm, failing to manifest in any appreciable way its potency to transform into a lens. Transplanted conjointly with the optic vesicle, it develops into a beautiful lens every time the contact between the two remains undisturbed.

So far the lens ectoderm has not exhibited a potency in any respect different from that of the surface ectoderm in general. To both, in equal measure, belongs the potency to form lens-like pearls. Should the capacity to form a real lens prove to be but a modification of this same potency, brought about by definite conditions, and differentiation of lens ectoderm into a lens be but a partial expression of a general potency of the surface ectoderm, will surface ectoderm differentiate into a lens if in contact with an optic vesicle?

V. SURFACE ECTODERM IN CONTACT WITH OPTIC EVAGINATION.

As referred to in section IV, displacements of embryonic structures often take place in grafted blastoderms. Optic vesicles may grow away from the ectodermal region against which they normally remain applied, they may reach and apply themselves against any other part of the ectodermal surface, or may remain separated from it by masses of mesenchyme, muscle, or cartilaginous tissue.

Lenses may be found in grafts at short distances (30μ to 40μ) from each other, separated by a loose mesenchymal tissue. Figures 3 and 4 represent two lenses which developed in one graft, each in close connection with a corresponding optic stalk. Figure 3 shows one of these lenses surrounded at one of its poles by convolutions of the optic stalk; figure 4 shows the other lens, which, like the first, is in intimate contact with an optic stalk, as may be seen in a study of the series. Unfortunately, it is not possible to show in one figure how close together these two lenses are. Only four sections, 7.5μ thick, separate them. The two corresponding optic stalks, however, emerge in this case from parts of the brain situated widely apart. One of the optic stalks is short and forms a compact structure, the other has approached the region of the former by growing away from its place of origin and can be followed from section to section in the form of a long, narrow, winding tube.

It is difficult to determine, in the case of optic stalks, how extensive this displacement is in relation to lens ectoderm, and which parts of the ectoderm the optic stalk has reached in its new location. It seems safe, however, to conclude, on the basis of a study of numerous grafts, that in a number of cases the optic stalk has come in contact with a part of the surface ectoderm which is different from that against which it would have been applied in its normal development. And, in response to an intimate contact with the optic vesicle, the surface ectoderm produces a lens which, in structure, is identical with the normal eye lens.

In my preliminary report on grafts (1924, the publication of which was delayed by the death of Oscar Hertwig), I stated that if contact between optic stalk and ectoderm continued after the formation of one lens, another would be formed. At that time I was inclined to take a lens-like, pearl-like structure for a rudimentary lens. A closer study has convinced me of the necessity for discriminating between these two types of spheroidal structures. After a regular lens has been formed, the optic stalk is usually seen in contact with the lens at its surface farthest away from the ectoderm. Occasionally, however, some of the convolutions of the optic stalk remain in contact with the ectoderm, and in this case epithelial processes are seen to invaginate from the deep surface of the ectoderm and in some instances to form pearl-like structures.

I have to admit that in some grafts optic stalks may be seen in rather intimate contact with parts of the surface ectoderm without a lens having been formed. It is difficult to say in this case whether or not the contact in the graft had been recently formed and the ectoderm had not yet responded. Possibly such contact may produce its effect only at a definite stage of development, or perhaps not all of the surface ectoderm against which an optic stalk may be applied has the potency to differentiate into a lens.

On the other hand, no well-formed lens can be found in any of my grafts that does not show a well-established contact with the optic evagination, even after 7 days' growth of the graft. Contact with the optic evagination, once established, seems to be not easily lost. The loose mesenchyme of the allantois, or that of the grafted blastoderm, may proliferate, grow between the lens and ectoderm, and finally separate them; but the contact between optic vesicle and lens persists in a very large majority of cases, fortunately for the student of this problem; for if this contact were dissolved by ingrowing mesenchyme, one would have difficulty in telling whether or not contact had been established at some previous time. It is rather remarkable that in grafts in which lenses have developed in an irregular way, even though they sometimes attain a rather large size, the contact with the optic evagination is usually found to be a short one.

Among my large number of grafts there is only one in which a rather small irregular lens is found close under the ectoderm, separated by large masses of mesenchyme from the optic stalks. Figure 5 represents a section from a graft of the cephalic part of a blastoderm of 8 somites, severed from the caudal part close behind the optic evagination. Both optic evaginations have developed into masses of coiled pigmented epithelium which emerge from the brain a short distance apart. The brain cavity has closed and is filled with blood-cells, normal in structure. The two optic stalks are found at about equal distance from the small, irregular lens, which is separated from them by mesenchymal tissue. Has there existed a contact between the lens and one of the optic evaginations? And if so, which one of the two was applied against the ectoderm? Only the first question can be answered with any degree of certainty. There *had* been a contact between ectoderm and optic evagination, for isolated groups of pigmented cells can still be found among the mesenchymal cells in close proximity to the lens. They are identical in structure with the pigmented epithelial cells which form the large coiled masses. They must have been at one time an integral part of these and were cut off from one of the optic stalks, when the latter became separated by the proliferating mesenchyme, and gradually pushed aside. At the stage at which the graft was fixed it was not possible to say which one of the optic stalks had been in contact with the lens ectoderm.

All of the observations and results of the various experiments seem to harmonize well; a lens differentiates at the expense of the ectoderm when a distinct and continued contact between it and the optic evagination is established; we observe it in normal development, it takes place in an allantoic graft of lens ectoderm and optic vesicle, and again in a case of blastodermal graft in which the optic vesicle has been displaced. Moreover, I have no observation which might throw doubt upon the necessity of this association for lens differentiation.

VI. GROWTH OF ISOLATED PRIMORDIA IN A HUMAN EMBRYO.

Transplantation experiments with isolated tissues and primordia of human embryos are fraught with great difficulties. Stunted and defective human embryos, whenever available, should be studied with the greatest care, for results not unlike those obtained by tissue culture may be observed in a number of them.

A young stunted human embryo from the Carnegie Embryological Collection has been placed at my disposal by Dr. G. L. Streeter. It is of great interest in that it shows fairly well-developed lenses, whereas other vital organs have either not appeared at all or are so rudimentary as to make their identification dubious. I agree entirely with Dr. Streeter's idea that the embryo must have developed following a defect in the primitive streak in the region of Hensen's node. After study of a great many allantoic grafts, in which definite parts of chick blastoderms have been transplanted, I should conclude that an extensive destruction in the region referred to had taken place at a time when the head process had already appeared. No notochord has been found in the specimen. Since this organ seems to be one which appears most regularly in chick blastodermal grafts from the head process stage on, it would seem that destruction must have taken place also within the region of the head process, and that only the most cephalic part of the head process continued to develop. An interesting feature of this embryo is the fact that the entodermal primordia failed to develop. Whether this is to be taken as an evidence of the extent of destruction or of its precocity is difficult to say.

Figure 6 represents a reconstruction of the whole embryo and shows clearly how defective it is. Nothing but the cephalic part of the medullary tube has developed and, as seen in figure 7, this development has resulted in the formation of a rudimentary brain, distinctly bent, its cavity closed and rudimentary optic evaginations formed. The embryo nevertheless possesses two easily recognizable lenses. They differ from lenses in their structure, but have developed far enough to become distinct from the surface ectoderm. Some of their cells have transformed into elongated lens fibers. Neither of the two lenses has been entirely overgrown by and separated from the ectoderm. They seem not to have been properly invaginated and one of them is protruding over the outer surface of the ectoderm. On both sides the lens has developed in a region where normally one would expect lens ectoderm to be situated. The optic evaginations, rudimentary as they appear to be, are separated from the lenses by strands of mesenchyme, and no contact exists between the two primordia. Does this mean that no contact ever existed, and that in this specimen the lenses are products of self-differentiation of the lens ectoderm? I do not think so.

The lens ectoderm, as found in this embryo, did not undergo its development as an isolated primordium. Brain and optic evaginations are present and this would indicate that the early development in the cephalic ectoderm was normal and that invagination and evagination processes have been regulated in this layer, as they would have been under normal conditions. At the stage reached by this embryo, large strands of mesenchyme are separating the two primordia.

Mesenchymal tissue is present in large amount. Except for the brain vesicle, the embryo stump should be described as a mass of mesenchyme within an ectodermal bag. The mesenchymal tissue is peculiar in structure. Typical fusiform or stellate cells, with clear and oblong nuclei and distinct nucleoli, are present in considerable numbers, but by no means constitute the predominating elements in the tissue. A great number of free round cells are found, either accumulated in groups or dispersed between the typical mesenchymal cells. The nature of these cells can not well be determined. Of the size of polymorphonuclear leucocytes, they contain nuclei broken up into parts, 4 to 6 in number. Judging by the well-preserved state of the syncytial and ameboid cells that form the core of the villi around the embryo, the karyorrhexis in the ameboid cells within the embryonic body probably took place while the embryo was alive. The picture is characteristic of an acute inflammatory process.

There is another peculiar feature characterizing the mesenchyme of this embryo. This is the absence of vessels and of erythrocytes. On the other hand, large and irregular spaces, with a clear exudate but no distinct endothelial lining, are present. In some places may be seen strands of fusiform cells simulating growing capillaries, but with no vessels as their origin.

The lenses and optic evagination are separated by spaces occupied by clear exudate, typical mesenchymal cells, and a great many free ameboid cells of the polymorphonuclear (?) type. No time can be fixed at which these products appeared between the two primordia and separated them. The normal development of the ectodermal primordia in the early stages seems to indicate that they have been separated at a relatively late stage. The polymorphonuclear cells could not possibly have developed within the embryo, no hemopoietic centers being present. They must have been brought into it from the maternal tissues and, fortunately, a number of maternal vessels can be discovered in the specimen, which along with fully differentiated non-nucleated maternal erythrocytes, contain many polymorphonuclear leucocytes. Without giving the certitude of direct observation, the analysis of the results brought about by the pathological development in the human embryo confirms rather than disproves the conclusions reached by the study of the chick blastoderm grafts. A contact between lens ectoderm and optic evaginations had evidently existed in this embryo and lens formation had started. At this time the mesenchymal growth, the round-cell infiltration, and the exudative processes took place and separated the two primordia. The lenses, not completely detached from the surface ectoderm, remained partly incorporated in this membrane. Both lenses are found tightly inclosed by the ectodermal layer. One of them has not been quite overgrown by the ectoderm and bulges over the surface. Has defect in lens formation in this case been brought about by insufficiency in the correlation between the two primordia, established but not maintained?

VII. DISCUSSION OF CORRELATION BETWEEN LENS ECTODERM AND OPTIC EVAGINATION.

The necessity of contact between lens ectoderm and the optic evagination has been established by the series of experiments and observations described. There seems to be no lens formation without such a contact; on the other hand, a contact between the optic vesicle and a part of the surface ectoderm in general may bring about lens formation. Lenses developed in grafts may be perfectly normal in structure; in a number of cases, however, they are irregular in shape and in the arrangement of their cells, and smaller in size, approaching the epithelial pearl in appearance. In most of these cases contact between lens and optic stalk can still be demonstrated. It would seem, therefore, that mere contact is not sufficient to call forth in the ectoderm differentiation of a normal lens.

Should the stage of differentiation reached by the optic vesicle while the contact is being established be considered of import for the effectiveness of the correlation? I do not think so. Young primordial optic vesicles may undergo a peculiar transformation in grafts, as described in section IV. However irregular their differentiation may be, perfectly normal lenses develop in association with them. An analysis of the contact effected between optic stalk and defective lenses brings forward a character regularly observed in the formation of a defective lens—the contact is only for a short distance. In two cases (fig. 5, chick embryo; figs. 8, 9, human embryo) the contact which apparently at one time existed has been dissolved.

A clear picture of contact between lens ectoderm and optic evagination during lens formation in grafts is given in figure 10. Two similar invaginations of embryonic ectoderm are present above a mass of convoluted brain tissue. The graft, which included the cephalic part of the blastoderm with both optic evaginations, is only 2 days old. Pigment granules have begun to develop, however, in both of the optic vesicles and help greatly to identify them. One of the optic vesicles is closely applied to one of the ectodermal invaginations; only a virtual space exists between them; the contact, being of the nature of adhesion, is intimate and extensive. Whether the two ectodermal invaginations are occasional pleats of the ectodermal membrane is difficult to say. Though similar in shape, only one of them—that which is in contact with the optic evagination—is being transformed into a lens. Its separation from the ectoderm is almost effected, the mesenchyme gradually overgrowing it. The cells, formerly a part of the surface ectoderm of the embryo, are now part of a closed cavity whose walls are closely applied against each other. Elongation of the cells has begun and is more marked in the layer which is in contact with the optic vesicle.

Every analysis of normal and defective lens formation seems to emphasize a necessity for an intimate and extensive contact between the two primordia. Whether or not an action of chemical nature is involved in the correlation is difficult to say. Defective lenses seem to be formed every time the contact has been short, either in duration or in space.

The sequence of a contact effected is invagination of a part of ectoderm and differentiation of the ectodermal cells. Neither the one nor the other of the two processes seems to be specific. Invagination of a part of the ectoderm leading to its incorporation into mesenchyme may be effected either through the agency of the optic vesicle or by means of mechanical propulsion. There is no difference in the reaction of lens ectoderm and surface ectoderm in general to either of the two agents. A lens is differentiated in the first case, a pearl in the second, by both lens ectoderm and surface ectoderm. Formation of solid structures seems to be a natural sequence of an incorporation of an epithelial membrane into mesenchyme involving elongation and rearrangement of the epithelial cells. Although similar in nature, the differentiation process in lens and pearl formation differs greatly in the degree of regularity attained by the structure. In lens formation an organ results which, in a normal embryo, seems to be wonderfully adapted to its function, and which, in a graft of the allantois, exhibits the same regularity in the most minute details, even though situated where it can never function.

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PLATE 1.

FIG. 1. Transplant of eye primordia (optic vesicle and lens ectoderm) in the allantois, 3 days' growth. Optic stalk is in the form of a convoluted mass of pigmented epithelium merging with transplanted portion of brain tissue. Lens moved away from ectoderm and overgrown by the convolutions of the optic stalk.

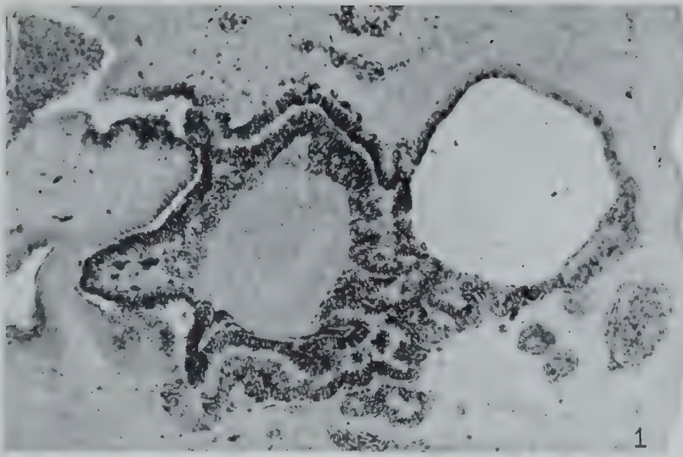
FIG. 2. One of two lenses (see fig. 4) developed in one graft of the cephalic portion of a blastoderm. The lenses are situated close to each other (see text), separated by a slender strand of mesenchyme. This lens, with well-developed fibers, was found in intimate contact with optic stalk in succeeding sections of this series.

FIG. 3. Transplant of eye primordia (optic vesicle and lens ectoderm) in the allantois, 5 days' growth. Lens and optic stalk in intimate contact.

FIG. 4. One of two lenses developed in one graft (see description of figure 2) of the cephalic portion of a blastoderm. Lens overgrown at one of its poles by the optic stalk.

FIG. 5. Two optic stalks and one lens developed in a graft of the cephalic portion of a blastoderm. The optic stalks emerge from the brain a short distance apart. Lens separated from both by a large mass of mesenchymal tissue. Isolated groups of pigmented epithelial cells, however, are in close proximity to the lens, as disclosed by high power. The contact of lens previously established with one of the optic stalks no longer exists.

FIG. 6. Lens primordium and optic evagination in intimate contact during the process of lens formation.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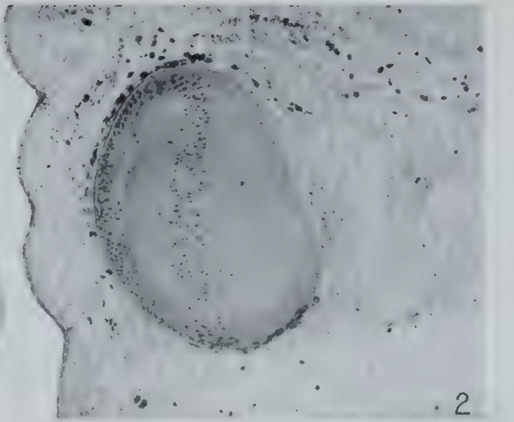
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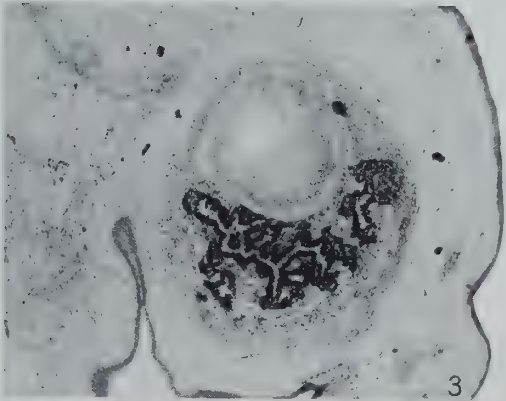
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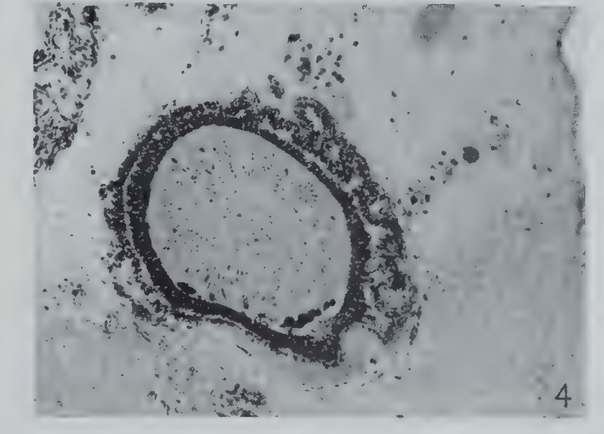
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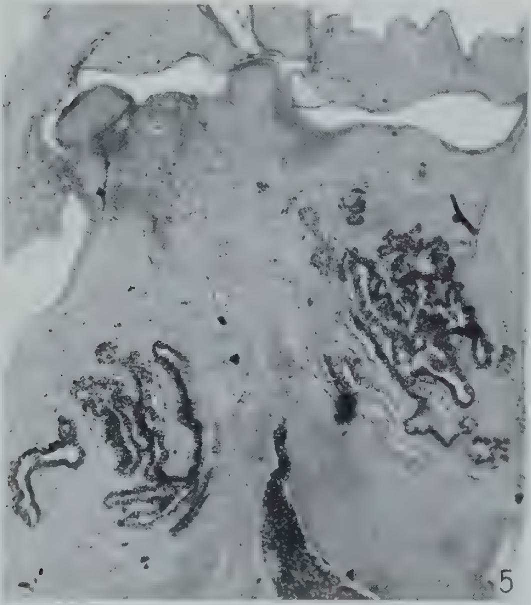
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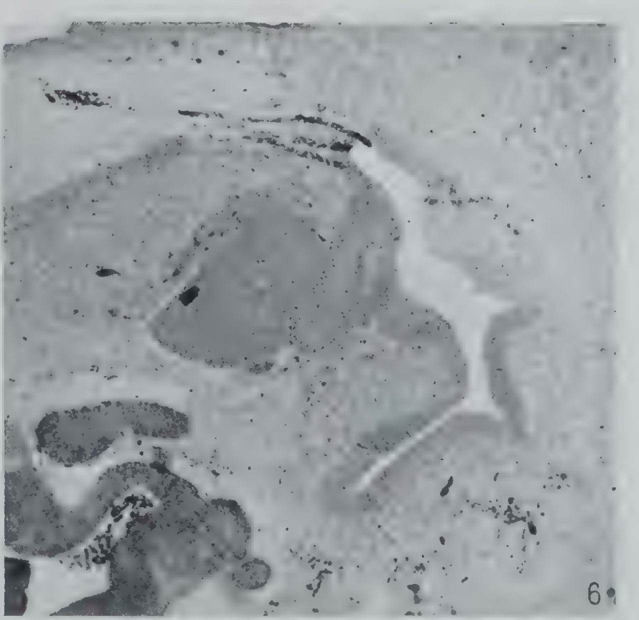
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PLATE 2.

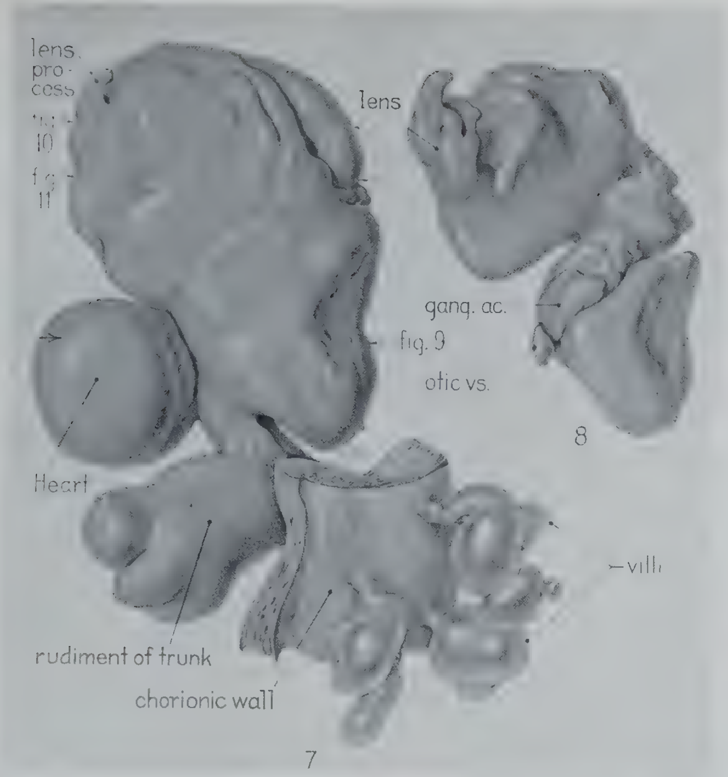
FIG. 7. Drawing of a reconstruction of a markedly stunted human embryo (Carnegie Collection No. 4211). A portion of the chorionic wall is shown, to which the embryo is attached by a short body-stalk. One can recognize the head portion, heart region, and a remnant of the trunk. A process from the lens projects from the left side of the head region.

FIG. 8. Reconstruction of the central nervous system of the head region of the same embryo. The irregular left lens can be seen, and from a bulging portion of the forebrain region there is a process which appears to be the rudiment of an optic stalk. In the hindbrain region one can make out the acoustico-facial ganglion and a small otic vesicle with which it lies in contact. Figures 7 and 8 are reproduced at the same magnification, 50 diameters.

FIG. 9. Section through hindbrain region, showing two otic vesicles and, in the upper part of the figure, the heart region. ×100.

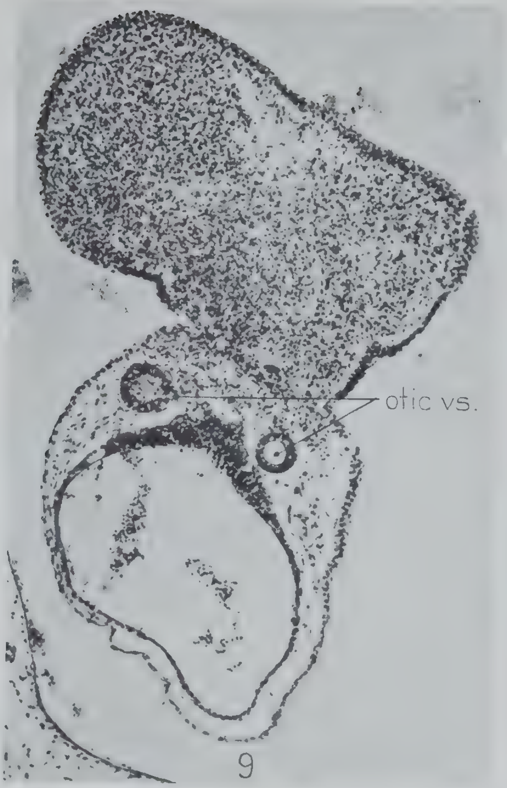
FIG. 10. Section through the upper part of the left lens, showing its extrusion through the surface of the head. The more regular plate to the right resembles retina, but in lower sections it can be traced directly into the rest of the lens. ×216.

FIG. 11. Section through the main portion of the left lens. Near it is the wall of the central nervous system, showing below a process which appears to represent the optic stalk. ×216.


lens process
fig. 10
fig. 11
lens
gang. ac.
fig. 9
otic vs.
Heart
villi
rudiment of trunk
chorionic wall

7

8


otic vs.

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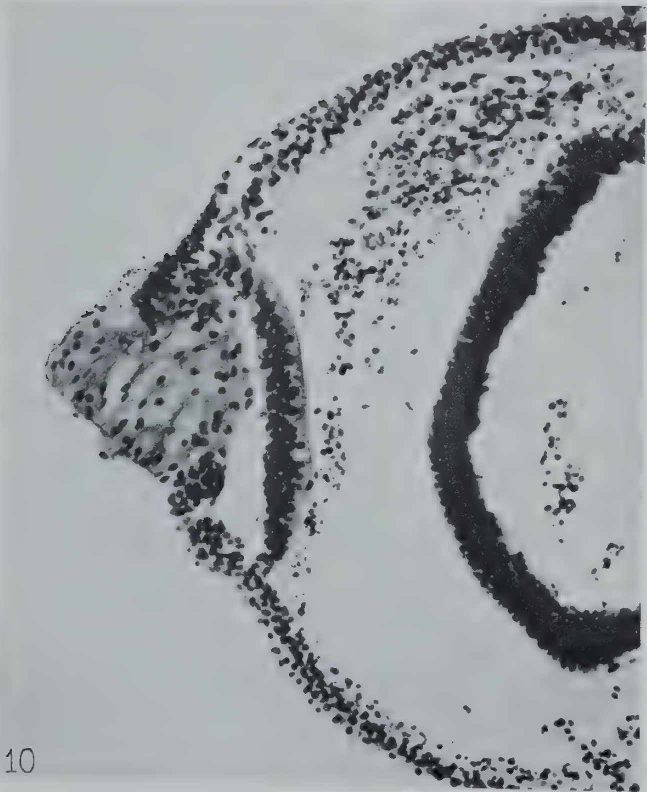

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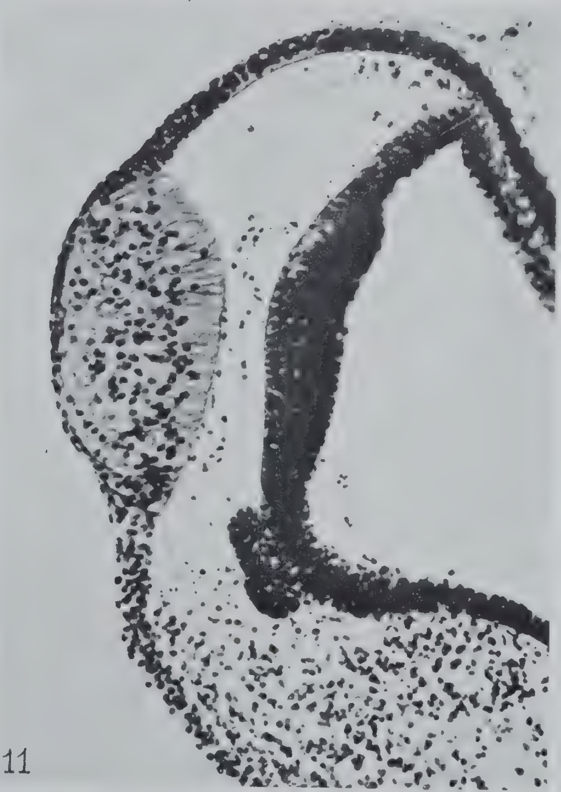

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CONTRIBUTIONS TO EMBRYOLOGY, NO. 95.

MENSTRUAL RECORDS AND VAGINAL SMEARS IN A SELECTED GROUP OF NORMAL WOMEN.

By Jessie L. King,

Department of Physiology and Hygiene, Goucher College,
Baltimore, Maryland.

With four figures.

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MENSTRUAL RECORDS AND VAGINAL SMEARS IN A SELECTED GROUP OF NORMAL WOMEN.

INTRODUCTION.

In the course of some investigations in this laboratory, a number of menstrual records have been collected from college women in normal health. Most of these records include ten or more consecutive cycles and in some cases the duration of the period is given. Vaginal smears were made daily by seven members of the group covering from two to three cycles for each. This paper includes these statistics and observations which, although small in number and based on data from a selected group, should prove of value when correlated with other studies.

MENSTRUAL RECORDS.

The difficulties in the way of the investigation of the human reproductive cycle and the need for more accurate information bearing on the problem are emphasized by a review of the literature on menstrual statistics. These seem to have been compiled from the reports of patients who presented normal menstrual histories, and the 28-day interval has "from time immemorial been accepted as the standard fixed by nature." (Kelly, 1908.)

Since there is general agreement in the statistical studies, it is necessary to cite only a few. From the examination of 1,000 case records in 1908, Kelly found that 942 women had reported 28 days as the interval, and 22 of the remainder gave 21 days. Sanes (1916), in studying 4,500 records, attempted to avoid the invariable inaccuracies in such data by taking, wherever possible, a second history, and he found that the two frequently disagreed in some details, especially concerning the interval. According to this data, however, 75 per cent of the subjects menstruated regularly, and of these 72 per cent at intervals of 28 days, 3.8 per cent at 30 days, and 3.3 per cent at 21 days. He says 3 to 4 weeks is the most common irregular interval. Still more recently Heyn (1920) gives 63.5 per cent of 1,684 cases as belonging to the 28-day type, 14.1 per cent to the 3 to 4 week type, and 10.9 per cent to the 3-week group. He states that the interval varies with the same individual. Marshall (1921), in a general discussion of the work done, gives from 28 to 30 days for the length of the cycle.

A brief report by Foster before the New York Clinical Society is of especial interest in relation to the data included in this paper. He had the actual records for 56 normal women which covered for each from 5 to 18 periods. In only 45 of the 380 periods was the interval 28 days. 225 were shorter and 110 longer. The extremes were 46 and 16 days. He states that the intervals varied in 4 cases from 25 to 29 days, in 3 from 21 to 30 days, and so on, in a table for the 56 subjects. Only one case showed perfect periodicity—26 days. This report seems to have occasioned much surprise among the members of the society. They regarded such irregularities in the intervals as due to some pathological condition of Foster's subjects, in spite of his assertion to the contrary.

There is general agreement among various writers that the usual duration of the menstrual period is from 3 to 5 days. The surprising inaccuracy of reports given from memory, even by intelligent women, is repeatedly referred to. Doubtless a day or two earlier or later than the accepted 28 is considered unimportant and the reports of these hundreds of cases must be approximately correct.

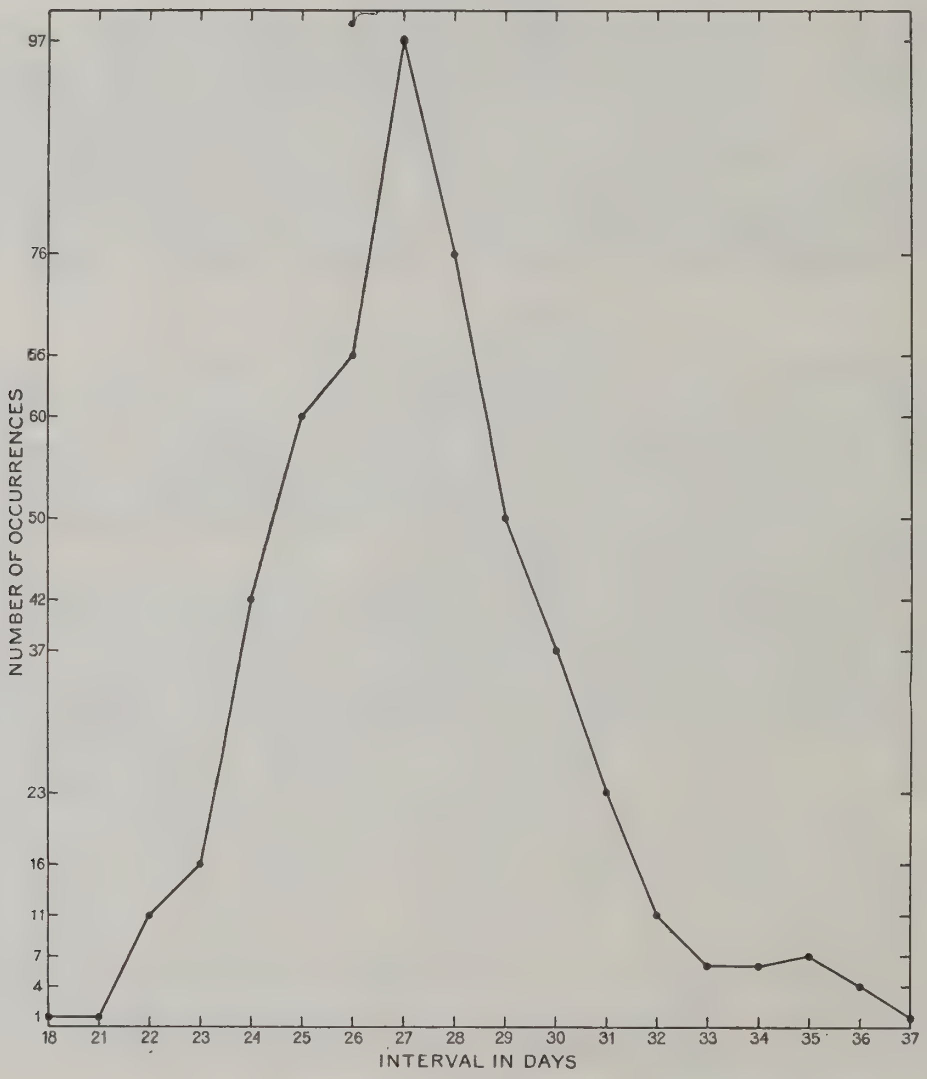

FIG. 1.—Frequency distribution curve of the menstrual intervals of 17 subjects. Intervals longer than 37 days are not shown.

The newly awakened interest in the mammalian sexual cycle, however, makes any accurate data on the human menstrual intervals of value. Although appreciating that the number of cases is far too small to establish averages, these records are presented in detail with the assurance of their accuracy. The data on duration of flow are included because they are interesting so far as they go. One subject (G) has intermenstrual pains usually followed by a mucoidal discharge. This condition is not found frequently (Croom, 1896; Kelly, 1908; Novak, 1921). Its occurrence in relation to the menstrual period is recorded for one individual by Kelly. Unfortunately, the record for the subject reported on in this paper is somewhat broken. It will be considered again in the second part.

A brief statement regarding each subject is given in the following protocols. The dates of the occurrence of the periods are indicated in Figure 1. Table 1 summarizes the data of the chart for the individuals and for the group. The duration of the periods is also tabulated (Table 2). The frequency distribution curve is given for the length of the cycles of the group (fig. 2) and separate curves for the individuals (A to D) having the largest number of recorded intervals (fig. 3).

TABLE 1.—*Menstrual intervals of seventeen subjects and number of occurrences.*

Interval in days.	Number of Occurrences.																	Total.
	A	B	C	D	E	F	G	H	J	K	L	M	N	O	P	Q	R	
18	....	....	....	....	....	....	....	....	....	....	1	....	....	....	....	....	....	1
21	....	....	....	....	....	....	....	....	....	1	....	....	....	....	....	....	....	1
22	....	9	....	....	....	1	....	....	....	1	....	....	....	....	....	....	....	11
23	....	10	....	....	2	2	....	....	....	1	....	....	....	....	1	....	....	16
24	1	27	4	....	1	7	1	....	....	....	....	....	....	....	2	....	....	43
25	2	28	13	....	1	7	....	3	....	....	2	....	1	....	2	....	1	60
26	9	14	17	....	3	6	3	6	1	2	2	....	1	1	1	....	....	66
27	28	3	20	1	11	4	7	8	....	2	4	2	2	3	....	1	1	97
28	23	2	6	8	15	2	8	3	....	1	3	2	1	2	....	....	....	76
29	13	....	2	14	7	....	1	2	2	1	....	5	1	....	1	1	....	50
30	13	....	....	11	6	....	2	....	....	1	....	....	....	....	1	2	1	37
31	6	....	....	6	1	....	....	....	4	1	....	1	1	3	....	....	....	23
32	3	....	....	5	....	....	....	....	3	....	....	....	....	....	....	....	....	11
33	2	....	....	1	....	....	....	....	2	1	....	....	....	....	....	....	....	6
34	1	....	....	1	....	....	2	....	1	....	....	....	1	....	....	....	....	6
35	3	....	....	1	....	....	1	....	1	....	....	....	1	....	....	....	....	7
36	....	1	1	....	....	....	....	....	1	....	....	....	....	1	....	....	....	4
37	....	....	....	....	....	....	....	....	....	....	....	....	1	....	....	....	....	1
41	....	....	....	....	....	....	....	....	1	....	....	....	....	....	....	....	....	1
43	....	....	....	....	....	....	....	....	1	....	....	....	....	....	....	....	....	1
44	....	....	....	....	....	....	....	....	1	....	....	....	....	....	....	....	....	1
46	....	....	....	....	....	....	....	....	1	....	....	....	....	....	....	....	....	1
47	....	....	....	....	....	....	....	....	1	....	....	....	....	....	....	....	....	1
49	....	....	....	....	....	....	....	....	....	1	....	....	....	....	....	....	....	1
53	....	....	....	....	....	....	....	....	1	....	....	....	....	....	....	....	....	1
Total	104	94	63	48	47	29	25	22	21	13	12	10	10	10	8	4	3	523

TABLE 2.—*Duration of menstrual periods of ten subjects and number of periods.*

Duration in days.	Number of Periods.											Total.
	B	C	D	F	G	H	J	M	N	P	R	
3	......	......	......	......	22	1	1	4	......	6	......	34
4	4	3	1	......	8	5	3	4	1	5	2	36
5	21	10	9	......	1	12	3	3	8	......	......	67
6	9	28	3	2	......	4	......	......	......	......	2	48
7	......	11	......	2	......	......	1	......	......	......	......	14
8	......	2	......	......	......	......	......	......	......	......	......	2
Total	34	54	13	4	31	22	8	11	9	11	4	201

PROTOCOLS.

Subject A.—Aged 30 at the beginning of this record in 1908. She is 5 feet tall, weight 150 pounds. During the first two years she was keeping house, studying and traveling. In 1911, 1912, and 1913 she continued to keep house, did part-time teaching, and studied some. During the next 3 years most of her time was taken up with graduate study, while in 1917 she was a full-time college teacher. Her health was normal and she had no dysmenorrhea. The record includes 104 intervals, the greatest number being 28 and 27 days in length.

Subject B.—Age 29 years at the beginning of the record in 1916. Height 5 feet 5 inches, weight 135 pounds. She was engaged in scientific research which continued throughout the year. She had no dysmenorrhea, was an athletic individual and reported that the occurrence of the menstrual period made almost no difference in her activities. "For many years" following the first period, which came on her fourteenth birthday, the interval was 28 days almost to the hour and the flow continued for 7 days. During the 6½ years covered by this record, 28 of the 94 periods occurred at intervals of 25 days, and the longest, with one exception accounted for by a preceding operation, was 28 days. The duration of 21 of the 34 periods in which it was recorded was 5 days. She was married and her husband died during the period of the record.

The subsequent record of this subject, although available, is not included on her chart nor in the tables or graph. It covers 42 intervals—from January 22, 1923, to September 20, 1925, and the intervals have the following distribution:

Intervals in days.....	34	26	25	24	23	22	21	20	Total
No. of occurrences.....	1	1	11	9	10	7	2	1	42

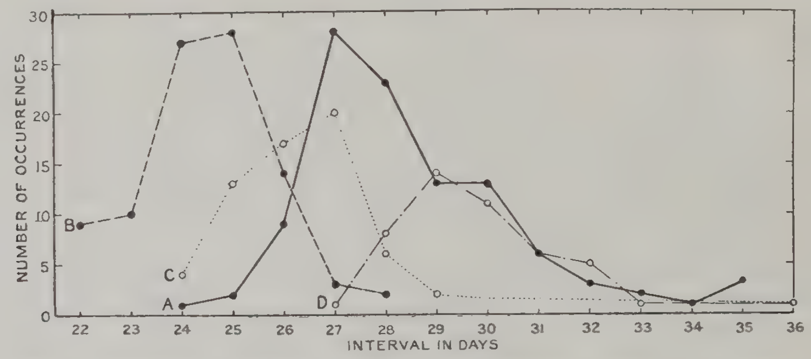

Fig. 2.—Frequency distribution curves of the menstrual intervals of subjects A, B, C, and D.

Subject C.—Age 21 years at the beginning of the record in 1915; height 5 feet 2 inches; weight varied from 100 to 114 pounds. She was a student and later a college instructor with much laboratory teaching. First menstrual period occurred at 11 years 11 months. The most frequent interval of the 63 cycles was 27 days. The flow continued from 4 to 8 days.

Subject D.—Age 23 years when record was started. Height 5 feet 3 inches, weight 92 pounds. She was engaged in laboratory teaching. Health was good but she had always had dysmenorrhea during the first day of the period; usually it extended through the second. The cramps, headache, and nausea were so severe that it was usually necessary for her to stay in bed for at least a day.

Periods began when she was 13 years old; 48 intervals are included in the record, the most frequent length being 29 days, which occurred 14 times. The duration of the period was recorded in 13 instances; in 12 of these it was 5 days.

Subject E.—The record began when she was 13½ years old, the occurrence of her first period. It is continued through 24 intervals and then, after 10 years, a second record including 23 intervals is given. Height and weight for the early period could not be obtained, but at the present time she is 5 feet 9 inches tall and weighs 160 pounds. She has some discomfort at the time of menstruation but not enough to interfere with the administration work in which she is

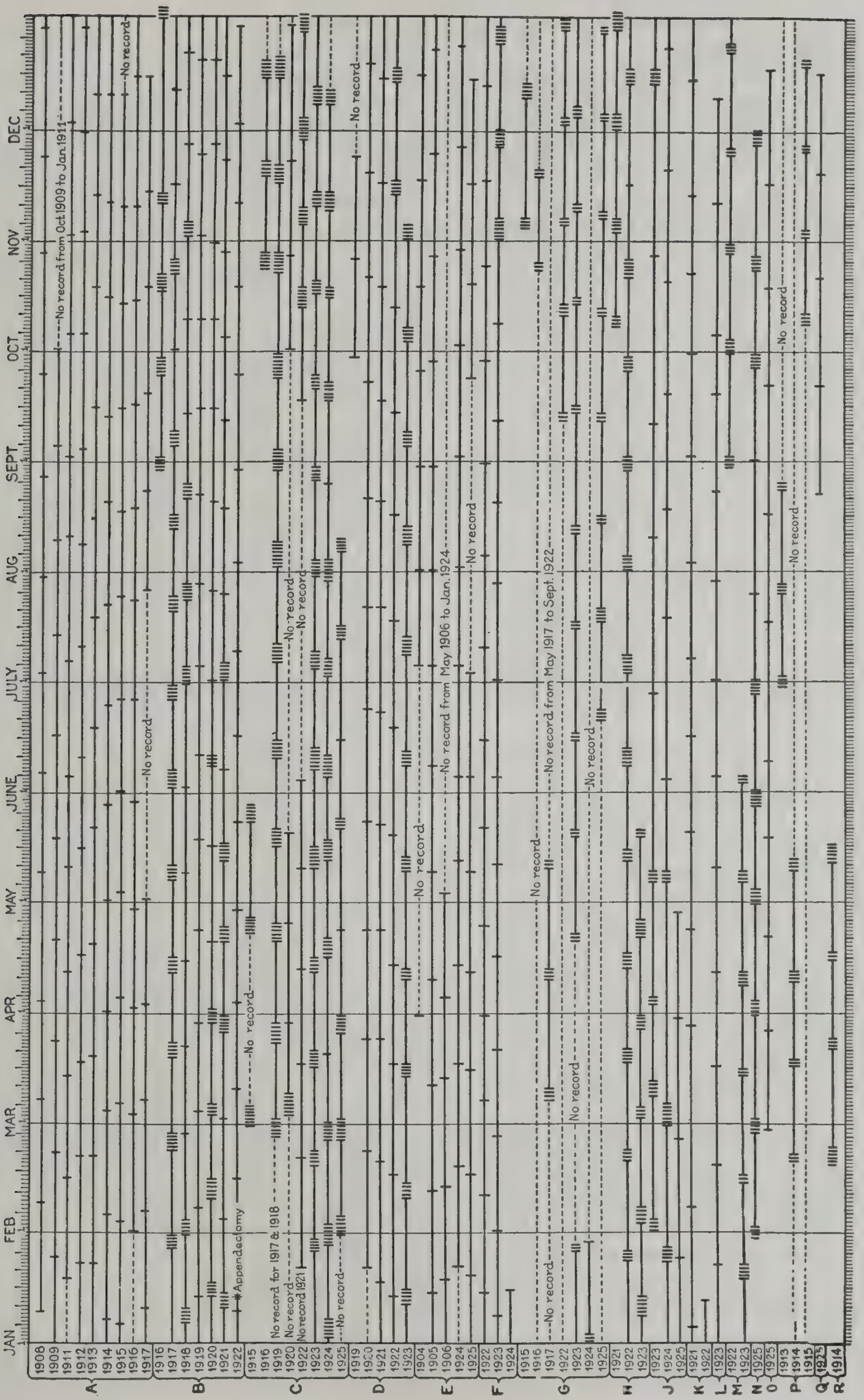

FIG. 3.—Dates of occurrence of menstrual periods in 17 subjects. 523 intervals are shown and in 201 instances the duration of the period is indicated.

engaged. The record includes 47 intervals, the most frequent being 28 days.

Subject F.—Age about 35. Health normal and no dysmenorrhea. She was carrying a heavy teaching schedule with laboratory work and, during most of the summer, was engaged in research work. Of the 29 recorded intervals 7 were 24 days and 7 were 25 days each.

Subject G.—Aged 27 years at the beginning of the record in 1915. Her height is 5 feet 3 inches and she weighed from 100 to 125 pounds. She was a college teacher. Her health was excellent but the menstrual periods were frequently preceded by headache and general depression. Since 1904 she has frequently, though not invariably, had intermenstrual pains about the middle of the interval. They are usually on the right side and there is a feeling of heaviness and soreness. A mucoidal secretion usually precedes the pain. A thorough medical examination failed to disclose any cause for it. The date of the mid-period was reported in 15 instances: three of these occurred 10 days after the menstrual onset, three 11 days after, three 12 days after, four times it occurred 13 days after and once each 14 and 15 days after. The first menstruation was in her thirteenth year. The record includes 25 intervals, the most frequent being 28 days.

Subject H.—Age 33 years at the beginning of this record; she was married and had two children. Health good. The first menstrual period occurred at 16 years. This report covers 22 intervals and of these 8 were 27 days; 5 days was the most frequent duration of the period.

Subject J.—Age 27 years at the beginning of the record in 1923. Height 5 feet 5 inches, weight from 130 to 150 pounds. Health was excellent and no dysmenorrhea. She carried a college schedule with much laboratory teaching and studied most of the summer. The first menstruation occurred in her sixteenth year. The record includes 21 intervals of variable length.

Subject K.—Age 25 years. She was an assistant in a college laboratory. Though not very strong she was in normal health and had no dysmenorrhea. The first menstruation occurred when she was 13 and at first the periods came at irregular intervals of from 5 to 6 weeks. The record includes 13 intervals of irregular length.

Subject L.—College student 24 years old. She was not strong and her college course had been prolonged because of ill health. She was married in 1924. Twelve intervals are recorded and of these 11 are between 25 and 28 days in length.

Subject M.—Age 27 years, height 5 feet 6 inches, weight 100 pounds stripped. She was a graduate student in science. Her health was excellent and she had no dysmenorrhea. The first period occurred when she was 12½ years old. She was married in June 1923, and now has one child. The record includes 10 intervals, and of these 9 are from 27 to 29 days.

Subject N.—Age 35 years, height 5 feet 3 inches, weight 124 pounds. She is engaged in graduate study. Her health is excellent and she experiences no discomfort during her menstrual periods. They started in her fifteenth year. The record includes 10 intervals of variable length.

Subject O.—Age 19 years, height 5 feet 1 inch, weight 83 pounds. She is engaged in clerical work. Her health is normal but she usually has great discomfort on the first day of her menstrual periods. They started in her thirteenth year. Her record covers 10 intervals of variable length.

Subject P.—Age 32 years at the beginning of the record in 1913; weight 110 pounds, height 5 feet 7 inches. She was in normal health and had no dysmenorrhea. The first period came in her thirteenth year. The 8 intervals recorded are of varying length.

Subject Q.—Age 27 years, height 5 feet 4 inches, weight 122 pounds. She is a college instructor with much laboratory teaching. Her health is excellent and she has never had any dysmenorrhea. Her record includes 4 intervals.

Subject R.—Age 22 years. She was a college student, strong and athletic. She experienced no discomfort at the menstrual periods. The first occurrence was in her fourteenth year. Her record covers 3 intervals. She was married a few years after this record was made.

VAGINAL SMEARS.

While interest in the human reproductive cycle centered for many years about the menstrual period, the time when ovulation was supposed to occur, the statistical studies on the cyclic structural changes in the corpus luteum and uterine mucosa have served to clarify ideas regarding the significance of menstruation (Hitschmann and Adler, 1913; Schroeder, 1914; Shaw, 1925). Furthermore, a better understanding of the problems of human reproduction has been made possible through the important comparative investigations of the œstrous cycles of mammals.

Since the literature bearing on this subject has been reviewed recently by Evans (1922) and Corner (1923), with special reference to man, it is sufficient to consider only the present conception of the relation of ovulation to the other phases of the cycle. This conception, based on observations of the changes in the ovary and the uterine mucosa, assigns the usual 3 to 5 days to the menstrual flow. This is followed by growth of the follicle and maturation of the ovum. Ovulation probably occurs at the end of this interval, 10 to 15 days after the preceding menstrual onset. During this phase the endometrium, desquamated by menstruation, is reconstructed and grows vigorously. In the next phase the ovum migrates, the corpus luteum forms, matures, and after about the twenty-eighth day begins to retrogress. This initiates a new cycle, indicated by the beginning of menstruation. The present tendency is to place the time of ovulation at the earlier date—the tenth day. When the intervals are greater or less than 28 days, as is certainly often the case, it becomes even more difficult to approximate the date of ovulation than in the case of the 28-day interval.

Although the study of the human reproductive cycle has been approached from various angles, obvious difficulties make it impossible to obtain that exact knowledge of its phases which has been secured for some of the lower animals. The vaginal-smear method, introduced by Stockard and Papanicolaou (1917) and applied by them to the guinea-pig, by Allen (1922) to the mouse, by Long and Evans (1922) to the rat, and by Hartman (1923) to the opossum, has given a sharply defined picture of the sexual cycles of these animals. Corner (1923) used it in studying a primate, *Macacus rhesus*, and his results have an especial bearing upon the work reported in this paper. He found a slight tendency toward a rhythmical variation in the type of the vaginal secretion which is possibly comparable to that described for the mammals referred to above. The leucocytes did not suddenly disappear completely in the manner which indicates the time of ovulation in rodents, nor was there the massive desquamation of epithelial cells, followed by leucocyte infiltration as seen in these animals. Corner found, however, that in some regularly menstruating animals there were relatively few epithelial cells and relatively many leucocytes during the first half of the intermenstrual interval, a sharp drop in the number of leucocytes 10 to 15 days before the appearance of the next menstruation and that, during the latter half, there was an increased desquamation of epithelial cells. Autopsies were made on two animals that were killed on the assumption that ovulation occurred at the time of the sudden decrease in the number of leucocytes. The recovery of the ovum from the Fallopian tube of one animal and from the uterine cavity of another supported the evidence that it must have occurred 10 to 15 days after the onset of the last menstruation. There is a report by Lehman, 1921, on the examination of vaginal smears from clinical cases, in which he says that in normal adult women, free from gynecological diseases, the secretion contains epithelial cells alone or epithelial cells with a few leucocytes.

Recently, Papanicolaou (1925) published a preliminary report on the "Diagnosis of early human pregnancy by the vaginal smear method." This work, carried on during the past year, includes observations on normal women and states that a number of different stages have been distinguished in the secretion. Allen (1925) mentions that, though he and his collaborators have found a decided variation in the number of epithelial cells and leucocytes in the vaginal smears of women, there is not the clear-cut picture seen in the case of rats.

Three years ago, at Dr. Corner's suggestion, such an investigation was begun in this laboratory. Publication of the findings has been delayed in the hope that, from a larger series of observations, definite results could be obtained. They are reported now in the hope that they will contribute to a further knowledge of the subject, although far too meager to be of value if considered alone.

METHOD AND OBSERVATIONS.

It was necessary to find reliable subjects having sufficient scientific interest to be willing to make slides of the vaginal secretion daily for continued periods. Technique which could be used by the subjects made it impossible to obtain smears without bacterial contamination, but an effort was made to have them free from cells from the exterior. Not finding a method which could be used with ease and without discomfort, it became necessary to resort to a very simple procedure or to lose the cooperation of the group. A small metal probe with expanded concave end was used. By gently scraping the wall of the vaginal canal an appreciable amount of secretion could nearly always be obtained, such as could not be gotten from the freshly cleansed surface about the vaginal orifice. The secretion was frequently thick, adherent and hard to spread. Jenner's blood stain was used for all slides though many were restained later with Ehrlich's hematoxylin. Each complete series was kept for repeated examination and for comparison with those of other subjects.

In examination of the slides account was taken of the relative number of leucocytes and epithelial cells and of the character of each. Corner's method of rating the number on the basis of 8 as a maximum was used, since it was desirable to correlate these observations with those made on the monkey.

The ages of the seven subjects ranged from 26 to 41 years. All were in good physical condition and were engaged in academic work. At least three menstrual cycles were covered in five cases, although in one of these (G) they were not continuous. In each of the other two subjects, two menstrual periods, though not two complete cycles, are represented. The menstrual records of the seven subjects are included in the first part of this paper and the same designations are therefore used for them here—C, D, F, G, J, P, and Q. The menstrual histories are not repeated. The records of three subjects are charted and, for convenience of reference, these cycles are divided according to the usual method, into menstrual, postmenstrual, intermenstrual and premenstrual periods.

The record of D covers 96 days with the interruption of only two days (fig. 4).

A general comparison of the character of the cells and the ratio of epithelial cells to leucocytes in the corresponding phases of the three cycles can be made. For example, the leucocyte and epithelial cell-content is variable during the menstrual periods, the number of leucocytes is low on the first day of the postmenstrual phase and may continue so for 4 days, but is always followed by a rise, at which time there are many polymorphonuclear leucocytes. These, as well as the epithelial cells, stain distinctly. The intermenstrual smears present variable pictures. Cycle I, from a field swarming with polymorphs, changes on the thirteenth day after the last onset and the sixteenth day before the next, to one in which no leucocytes are present. A low level continues for 8 days. In Cycle II, while a drop occurs on the twelfth day after the last onset and 17 days before the next, lasting 2 days, it does not seem more significant than low ratios at other times. Again, in the three cycles the epithelial cells tend in the intermenstrual and premenstrual phases to form masses, and during the latter part of the time there is evidence of degeneration. Epithelial cells are frequently invaded by leucocytes and in some cases only bits of cytoplasm are clinging to the nuclei.

The record of the second subject, F, includes a little over three cycles—a total of 78 days (fig. 4). No regularity can be distinguished in the character of the smears at different periods. In the postmenstrual phase the epithelial cells are separate and have distinctly staining nuclei in contrast to the crumpled masses of cells which frequently characterize the days of the intermenstrual and premenstrual periods. If the third cycle were considered alone, the drop in leucocytes on the eleventh day after the last onset and the eleventh day before the next, might be regarded as significant. Though less marked, it is comparable to the first cycle of D, referred to above. However, the great irregularities in the ratios of the epithelial cells to leucocytes in the other cycles make it of doubtful significance.

The third subject, G, is especially interesting because she frequently had intermenstrual pains such as have been observed in a few cases. It has been suggested that their occurrence marks the time of ovulation. She had no dysmenorrhea, though she often had headache and general depression during the last 2 or 3 days of the premenstrual phase. At the mid-period she had a feeling of heaviness and soreness in the pelvic region and pain, usually on the right side, lasting from 12 to 24 hours. There was frequently a slight discharge before the pain. Eight of these periods are noted in figure 4. The record covers four complete cycles and parts of four additional ones, though they are not continuous.

During the different menstrual periods, the number of leucocytes and epithelial cells is variable; the secretion is scanty for a few days following, then

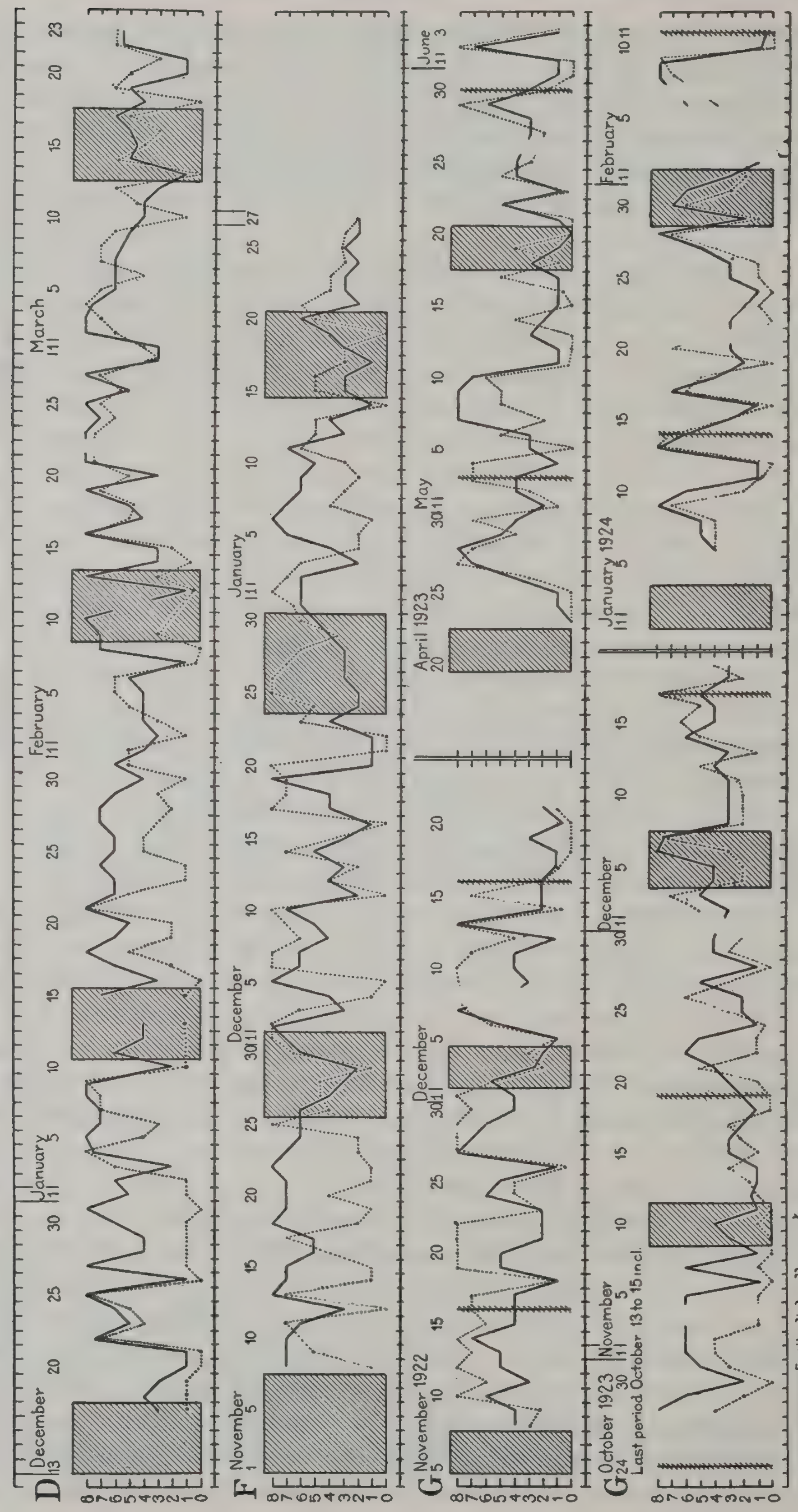

FIG. 4.—Graphs showing the proportionate relation of leucocytes and epithelial cells in the vaginal smears of subjects D, F, and G.

usually becomes more profuse, continuing so into the intermenstrual phase. During this period the epithelial cells are clearly outlined and the nuclei distinct; many polymorphs are distinguished and these may be closely packed among the other cells. On the days of the intermenstrual pains the subject's notes usually indicated that the secretion was profuse, a day or two afterward it was scanty and hard to get enough for the slide. Since the same condition was occasionally recorded on other days, special significance can scarcely be attached to it. A decrease at this time may be followed by an increase both in leucocytes and epithelial cells. The latter tend to form irregularly staining masses and both types may stand out more clearly in the late premenstrual phase.

The vaginal-smear record of C covers 96 days, with the omission of 2 days, and includes three complete cycles. C's chart is not included since it showed so little variation from day to day. The secretion was profuse except for a day or two following the cessation of menstruation and for 2 or 3 isolated days of the three cycles. With these exceptions, the leucocytes and epithelial cells were continuously near the maximum. The nuclei of the polymorphs usually stood out distinctly. During the late intermenstrual and the premenstrual phases, the picture is less definite and there is evidence of deterioration of the cells.

The record of Q extends over 51 days. Two complete cycles, lacking 3 days of one menstrual period, were included. The epithelial cells and leucocytes were always abundant, the latter never being rated below 4, and frequently both were at the maximum. Polymorphs crowded between the other cells and sometimes almost covered them. There were often sheets of epithelial cells. The postmenstrual decrease in the secretion, such as usually occurred in the other subjects, was scarcely noticeable in Q.

The slides of the sixth subject, J, were not continuous through a complete cycle, as her intervals were usually long. However, 86 days were included with two menstrual periods; 41 of these were consecutive days, then there was an intermission of one week, and then the series of 23 days. Another series covered 22 days. The majority of the slides showed an average number of epithelial cells and leucocytes were absent or present only in small numbers.

The observations for P were continued for 98 days and covered three consecutive menstrual periods. No consistent variations in the character of the smears could be observed. A scant secretion containing faint masses of epithelial cells predominated, though there were days when their nuclei were distinct and when there were many leucocytes. Since this subject was 41 years old and her menstrual periods occurred at irregular intervals, the record is of questionable value. Another series of slides was made by her three years after this record and when menstruation had completely ceased seven months previously. This record, covering 29 days, differed from all others in that the slides invariably contained much mucous. The epithelial cells were numerous and, for 20 consecutive days, they were distinctly nucleated. There were occasionally degenerated leucocytes. During the latter part of the period, polymorphonuclear leucocytes appeared and epithelial cells, apparently freshly desquamated, as well as those staining more faintly. Then in the last few days there were masses of faintly staining epithelial cells and degenerating leucocytes. This series has been described in detail because, except for the mucous, the pictures were such as were often seen in the smears of regularly menstruating women.

DISCUSSION.

It would be futile to attempt any generalizations on the basis of observations on the small series of subjects. However, the statistical records serve to emphasize a point not sufficiently stressed, that there are surprisingly frequent variations in the length of the cycles in mature normal women. The irregularities are not to be accounted for by season or occupation. Most of the subjects were leading active lives but were engaged in more intensive work during the college year. These records do not support the idea that the "interval of days between periods is most frequently some multiple of seven" (Novak), nor that the majority of women menstruate during the new moon, a theory which is mentioned only because it again appears in a 1924 publication (DeLee). The data on duration of periods support the accepted idea.

A consideration of the studies on the vaginal secretions suggests interesting comparisons within the group and possible correlations with Corner's findings on the monkey may be discovered.

The subjects can be roughly divided into those whose vaginal smears showed much daily variation (D, F, and G), those with leucocytes frequently absent or at a minimum (J and P), and those in which large numbers of leucocytes were almost invariably present (C and Q). The cycles were of average length in C, D, G, and Q, longer in J and P, and shorter in F.

In these series there is greater correlation of the type of secretion with changes in the ovary and uterine mucosa, as they are supposed to occur according to the present conception, than with the postmenstrual, intermenstrual and premenstrual phase. In general the secretion is more scanty during the first few days following menstruation and the cells show less degeneration during the first part of the cycle than in the late intermenstrual and premenstrual phases. The leucocytes are of the polymorph type, although an occasional mononuclear can be seen. There is a definite decrease in the number of leucocytes in the secretion and a relatively high content of epithelial cells in cycle I of D, on the thirteenth day after one onset and 16 days before the next. This fall in the leucocytes continues long enough to be outstanding. A similar condition, though much less striking, is seen in the third interval of F, beginning in the middle of a 22-day cycle. Probably this fall in leucocytes is comparable to the intermenstrual decrease noticed in some of Corner's monkeys and may bear a relation to the time of ovulation. One is tempted to see a similar relation in the other cycles of D and F and to attach significance to the depression of the curve of G, at the time of or near to the occurrence of the intermenstrual pain. However, the irregularities are so great that such an interpretation seems unwarranted. Only a longer series of observations and a larger number of subjects would make it possible to determine whether these two cases represented the rule and not the exception in normal women. This latter conception is further supported by the record of Q, who would be regarded as the best subject of the group from the physical as well as the temperamental standpoint. She was strong and athletic and led a very regular life as far as diet and exercise are concerned. While not phlegmatic, she was not easily disturbed emotionally. However, her series of slides showed no evidence of periodicity in the character of the vaginal secretion. Even the very general points noted were less clear in her case. It is rather surprising that in P, after complete cessation of menstruation, the vaginal secretion should show at different times such a condition as that found in menstruating women.

The two types of secretion described by Lehman for normal adults may be found in the same individual and in addition there is a type in which the content in both leucocytes and epithelial cells is high.

One is forced from this study to conclude that the cellular content of the vaginal secretion of the normal human female is exceedingly variable and is a doubtful index of changes transpiring in the ovary and uterus. The indications of periodic variation are even less evident than those found by Corner for the monkey. Considering the higher degree of specialization, this is the condition which might reasonably be expected.

There was no consistent variation in the vaginal smear at the time of the midmenstrual pain in the one subject in which it occurred. A change was especially looked for at this time, since it has been suggested as the period at which ovulation occurs.

SUMMARY.

The dates of the menstrual periods of 17 subjects, 16 of them college women, are given. They include from 3 to 104 intervals, 523 in all. The most frequent interval is 27 days, occurring 97 times. The records strongly support the statements regarding the variability in the length of the cycles. The duration of the periods is recorded in 201 instances. This varies from 3 to 8 days, the most frequent being 5 days, as found in 67 cases.

Seven members of this group made vaginal smears daily for periods covering two or more intervals for each. Seventeen complete cycles and in addition parts of a number of other cycles were included in the records. There is great variation in the cellular content of these series and the slight periodicity sometimes noted is not sufficient to lead to the conclusion that changes in the human vaginal secretion serve as an index of periodic changes transpiring in the ovary and uterus.

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TRANSFORMATION OF MONONUCLEAR BLOOD-CELLS INTO MACROPHAGES, EPITHELIOID CELLS, AND GIANT CELLS IN HANGING-DROP BLOOD-CULTURES FROM LOWER VERTEBRATES.

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With thirteen plates.

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TRANSFORMATION OF MONONUCLEAR BLOOD-CELLS INTO MACROPHAGES, EPITHELIOID CELLS, AND GIANT CELLS IN HANGING-DROP BLOOD-CULTURES FROM LOWER VERTEBRATES.

INTRODUCTION.

The transformation of the mononuclear blood-cells into macrophages, epithelioid cells and giant cells in birds and mammals has recently been described by one of us (M. R. Lewis, 1925) as taking place in hanging drops of whole blood and a preliminary account of this same process as it occurs in hanging drops of whole blood of the lower vertebrates has also been given (Lewis and Lewis, 1925). By this simple but effective technique the puzzling problem of the inter-relation between these cells seems to us to be quite conclusively settled. In order, however, that our results may be more effectively established we are giving in the present paper a more extensive account of the behavior of the blood-cells from lower vertebrates in simple hanging drops. Crucial experiments of this sort far outweigh interpretations based on fixed and stained material, or on living material where the intermixture of various tissues renders the field of observation so complex that the eye can not follow the changes with great certainty. In the experiments here recorded we employed the method first utilized by M. R. Lewis for the study of the blood of elasmobranchs, teleosts, amphibia, and reptiles. In these blood-cultures there developed from the mononuclear cells macrophages (clasmatocytes) similar to those found in the connective tissue, the liver, and the spleen, not only of these animals but of higher forms as well, and also epithelioid cells and giant cells precisely like those found in tuberculous lesions (Lewis and Lewis, 1925).

Before presenting our observations on the blood-cells, it might be well to state clearly what we mean by the terms *mononuclear*, *macrophage* or *clasmatocyte*, and *epithelioid* cell.

MONONUCLEARS.

The term *mononuclear*, as employed in these observations, is in agreement with the usual meaning ascribed to it by hæmotologists. It is a large white blood-cell with an oval nucleus, somewhat eccentrically placed, and often indented on the inner side. It usually has a centrosome, located near the center of the cell and surrounded by short mitochondria and a few granules which stain with neutral red. These bodies often have a radial arrangement about the centrosome. The cells are highly phagocytic and migratory; they have thin membranous pseudopodia and a considerable amount of cytoplasm in proportion to nuclear material. They are the same as the monocytes of Naegeli and of Sabin, Cunningham, and Doan, and the endothelial leucocytes of Mallory, Foot, and Permar. They are easily distinguishable from the granulocytes and small lymphocytes, but in most species there are blood-cells which appear to bridge the gap between the mononuclears and the lymphocytes.

MACROPHAGES OR CLASMATOCYTES.

Our use of the term "macrophage" or "clasmatocyte" does not differ from that generally indicated by other observers. These large, highly phagocytic, migratory cells are widely scattered throughout the body in the tissue spaces, lungs, spleen, liver and other organs as discrete individuals that never form distinct tissues, as do the connective-tissue and endothelial cells. The macrophages are distinguished by numerous granules, vacuoles and ingested materials, which have the property of staining with neutral red and certain other vital dyes. They may contain fat globules irregularly distributed in the cytoplasm. The nucleus is eccentrically located, near or at the periphery of the cell, and is sometimes difficult to see. The cells possess rather characteristic thin membranous pseudopodia, similar to those of the mononuclear blood-cells, which differ in extent according to the activity of the cell. The centrosome is usually more or less obscured by the large collection of cytoplasmic inclusions. The mitochondria are in the form of short rods and granules. The macrophages vary greatly in size and in the amount, character, and state of digestion of the ingested material. The ingested particles and the vacuoles that may develop about them also vary in size. All sorts of transition forms between them and the mononuclear blood-cells occur in the body. Any careful examination of the subcutaneous tissue, the lymph nodes, the spleen or the thymus gland reveals a series of cells ranging from the mononuclear to the largest macrophage. Such cells present almost every imaginable variation in size and shape, and also in the number, size and character of the included granules, vacuoles and ingested materials. Such a series, however, no matter how perfect, does not demonstrate in a conclusive manner the origin of either the macrophages from the mononuclears or the mononuclears from the macrophages.

Owing to their marked phagocytic powers they are able to take up all sorts of foreign materials and debris, such as bacteria or dead cells. They thus act both as protectors of the body and as scavengers. They digest this material and convert it into soluble form so that it can be utilized by the body or eliminated as waste products.

EPITHELIOID CELLS.

Epithelioid cells, usually supposed to be characteristic of tuberculous lesions, are found in other pathological conditions. One of the great problems in tuberculosis has been to determine the origin and nature of these cells. Fixed and stained sections of tubercles, where these cells are crowded together in dense masses, do not give an adequate idea of the real character of the individual cells. This can be gained best by a study of the living cells from tuberculous lesions. In conjunction with Dr. Willis (Lewis, Willis and Lewis, 1925), we examined in spreads the living epithelioid cells from tuberculous lesions of the rabbit's lung, both with and without the use of neutral red and janus green.

The living epithelioid cells vary greatly in size. They are characterized by a large prominent granular central area surrounding the centrosome. The granules composing this area have a marked affinity for neutral red and are arranged more or less radially about the centrosome, the largest ones usually occupying the periphery of the granular area. Surrounding this area in turn is an intermediate zone of mitochondria and fine granules. Fat globules often accumulate in this zone, especially in cells situated in the center of older tubercles. In frozen sections of the latter, the ordinary fat stains show them beautifully. The periphery of the cell consists of a clear hyaline layer, which often extends out into thin membranous pseudopodia similar to those of mononuclears and macrophages. The oval nucleus is eccentrically located in the intermediate zone and contains a more definite nucleolus than is found in the mononuclears. The living epithelioid cells of tuberculous lesions thus differ greatly in appearance from those seen in the usual fixed preparations. Fortunately, Dr. Willis has been able to devise a stain which shows some of their marked characteristics even in fixed material.

In the tubercles are many mononuclears and clasmatocytes, and in addition all stages between mononuclears and clasmatocytes, between mononuclears and epithelioid cells, and between clasmatocytes and epithelioid cells. Small epithelioid cells and all gradations between them and typical mononuclears also occur in normal tissues. They are easily recognizable in spreads with neutral red.

Although the existence of numerous intermediate stages between mononuclears and epithelioid cells suggests the origin of the latter from the former, it does not offer conclusive evidence for such a relationship. It is only when such cells are isolated and followed in the living state as in our experiments that one can be entirely certain of the relationship. It is this sort of proof that has been needed to clear up the question of the origin of the epithelioid cells and of the macrophages or clasmatocytes as well.

GIANT CELLS.

Many giant cells similar to those found in tuberculous lesions form in the blood-cultures. They are large multinucleated cells having the general characteristics of the epithelioid cell, except that their nuclei are considerably smaller.

These giant cells are of the Langhans type and, as in the case of the epithelioid cells, their structure can be appreciated best by an examination of the living cells of tubercles. They contain, as do the epithelioid cells, a large granular central area, which has a marked affinity for neutral red. Surrounding this is an intermediate zone containing granules, mitochondria, and often many fat globules. A peripheral zone of clear cytoplasm extends out to the border of the cell and is often prolonged into thin film-like pseudopodia. The nuclei lie in the intermediate zone or between it and the central area. Such cells were described by Lewis and Webster (1921) in cultures of tuberculous lymph nodes.

The term *lymphocyte* has also been employed in its usual meaning and includes cells somewhat variable in size. The term *granulocyte* offers no difficulty as to its meaning. It seems a pity, however, that the term *leucocyte* has come into such general usage as a synonym for the polymorphonuclear neutrophile, instead of retaining its older significance as a general inclusive term for white blood-cells.

Since the distinction between these cells depends to a great extent upon the presence of certain bodies within them and their behavior toward vital dyes, it seems advisable to discuss these matters briefly before describing our observations.

CELL INCLUSIONS AND VITAL DYES.

It has been known since the time of Ehrlich (1894) that various types of cells under certain conditions contain granules and vacuoles that have a marked affinity for neutral red—that is, become more deeply stained by neutral red than the surrounding fluid or medium in which the dye is dissolved. These bodies probably comprise a heterogeneous group of cell inclusions which vary in their origin and significance. Under normal conditions some cells, such as the ordinary fibroblasts, are entirely devoid of or contain but few bodies that stain with neutral red, while others, such as the macrophages or clasmatocytes, are often heavily laden with them. Under abnormal conditions (Lewis and McCoy, 1922) almost every type of cell may acquire such bodies. Various terms have been applied to them—for example, "grains et vacuoles de ségrégation" (Renaut, 1907), "granules and vacuoles" and "degeneration granules and vacuoles" (Lewis and Lewis, 1915; W. H. Lewis, 1919); Shipley (1919) considers them to be digestive vacuoles, and Evans and Scott (1921) introduced the term "segregation apparatus," a modification of Renaut's term.

Let us first consider the granules and vacuoles that arise from injury due to the cutting off of the proper oxygen or food supply or to the action of toxins, poisons, or waste materials. After the death of the animal, most of the cells in the intact body continue to live for hours, even days, and during this period accumulate varying numbers of granules and vacuoles that stain with neutral red (Lewis and McCoy, 1922). Most of these cells are, during the life of the animal, entirely free from such bodies and their accumulation in gradually increasing numbers after its death is probably the result of lack of oxygen or the accumulation of waste products from autolysis or some other process, resulting in the degeneration and finally in the death of the cell. Similar changes occur in the cells of older tissue-cultures, especially in those in the simple saline media free from dextrose (W. H. Lewis, 1919). Extensive vacuolization can be brought about in the course of an hour or two by the introduction of typhoid bacilli or their toxins into healthy cultures (M. R. Lewis, 1920). To call such bodies degeneration granules and vacuoles, as we have done, would seem to be in keeping with their significance in such cases, since they are neither ingested material nor vacuoles that have formed about ingested stuffs.

There are, in addition to the above, other granules which are derived from many sorts of ingested materials, such as red blood-cells, whole or parts of dead cells, bacteria, melanin and pigment granules, albumen, fibrin, and various inorganic substances, such as carbon, carmine, and trypan blue. Many if not all of the organic substances develop a marked affinity for neutral red and other vital dyes after they have been for a time in the cytoplasm and presumably are being digested. Vacuoles stainable with neutral red may form about them, as well as about the inorganic substances. Digestion of organic substances often proceeds as rapidly when the latter are naked in the cytoplasm as when they become surrounded by a vacuole (M. R. Lewis, 1923). Such vacuoles may be considered digestive vacuoles, although no digestive enzymes have been demonstrated in them.

It has been inferred by Shipley and also by Evans and Scott that ingested materials, both microscopic and ultra-microscopic, find their way into pre-existing vacuoles. We have never been able to confirm this, in spite of many observations on the ingestion, by various types of cells, of foreign material such as carbon, red blood-cells, dead cells, and bacteria. Our observations, and also those of Smith (1921) and of Smith, Willis, and Lewis (1922), where both the act of ingestion and the fate of the ingested particles were followed from minute to minute, show (1) that vacuoles are not present in most kinds of normal cells, (2) that all ingested materials enter first directly into the cytoplasm, and (3) that later vacuoles may develop around them. Evans and Scott did not thus follow the fate of ingested particles; as a matter of fact, the technique used by them does not permit one to determine whether or not ingested particles are taken into pre-existing vacuoles. Certain dye stuffs that are in solution, such as neutral red, brilliant cresyl blue, methelyne blue, and trypan blue, diffuse into granules and pre-existing vacuoles, often to such an extent that all the dye is removed from the surrounding medium and none left to stain vacuoles that may form later. On the other hand, when no vacuoles or granules are present, such dyes do not accumulate in the living cell unless they are clotted outside the cell and then ingested as solid dye particles. Some recent experiments by M. R. Lewis (1925) show that suspensions of fine carbon particles, and even trypan blue, when injected into the blood-stream, are almost instantly clotted there and rapidly ingested as visible masses by the blood-cells within the capillaries, especially in the lungs. Therefore, much of the segregation supposed to take place within the cell probably takes place outside of it and is dependent, not upon conditions within the cell, but upon conditions controlling the state of the colloidal solution itself. Very fine particles, whether they be dye stuffs (Evans and Scott) or carbon particles (Shipley), need no special segregation apparatus to account for their concentration after they are taken into the cell, since ordinary cytoplasmic currents afford sufficient means for this.

The dye stuffs that diffuse into cells stain only pre-existing granules and vacuoles; in other words, they stain only cell inclusions which are already present in the cell but which vary from time to time in character and amount in the same cells. The concentration of the vital dyes in these granules and vacuoles is not due to any special activity on the part of the cell, but is rather an accidental affair, due to the fact that the vital dyes, as they diffuse into the cell, form some sort of a physical or chemical union with the granules and vacuoles. Fat globules, if present in the living cell, will take up Sudan III, but no one would contend that they constitute a special segregation apparatus for this particular dye, nor would one consider that the mitochondria form a segregation apparatus for janus green. Consequently we consider that the term "segregation apparatus" gives an erroneous meaning to these granules and vacuoles.

TECHNIQUE.

The preparations, usually about 15 to 20 in each series, were made by drawing up a small amount of blood in a paraffin-lined capillary pipette directly from the heart, putting it, one drop at a time, on sterile coverslips and sealing the latter with vaseline rings on hollow ground slides. It is essential, of course, to use aseptic precautions throughout. The pericardial cavity was opened when the heart was still beating. The heart was usually held with sterile forceps as the slender pipette was thrust into the auricle or ventricle. Film preparations were usually made from the first and last drops, dried, and stained with Wright's stain. The blood-cultures from these cold-blooded animals were kept at room temperature and out of the strong light, as the latter has a decidedly injurious effect upon the cells.

OBSERVATIONS.

We endeavored to follow the changes occurring in the various types of cells seen in the living blood-cultures, in order to determine just which cells were transformed into macrophages, and into epithelioid cells and giant cells. A few days after the blood-cultures had been prepared, almost every one contained hypertrophied cells. Some cultures had all three types, macrophages, epithelioid cells, and giant cells, while in others we observed only one or two types. Fresh cultures were examined shortly after their preparation in order to identify the various types of white cells with and without the aid of neutral red and janus green, and to determine whether or not macrophages, epithelioid cells, and giant cells were ever present in the normal blood. In no culture did we find, during the first few hours, cells that we could classify under any of these three heads.

In many cultures, especially from amphibian and reptilian blood, there were often observed large numbers of small amœboid cells, some scattered and some in dense clusters. We are not quite clear as to their classification, but they probably correspond to cells that have sometimes been called spindle cells, thrombocytes or hæmoblasts. The blood clots were usually not very firm even at first, and in many forms soon became liquid.

It was fairly easy to follow the different types of white cells in the thin peripheral part of the drop, and in most cultures there was a clear peripheral edge of plasma, freé from red blood-cells, into which numerous white cells migrated. Here they often flattened out and afforded exceptionally fine objects for examination. It was more difficult to follow those cells in the deeper part of the drop, even when cells were on the cover-glass. In order to see the latter more clearly, the plasma drop, which often liquefied, was washed off with a few drops of Locke solution or sea-water, modified to suit the osmotic pressure of each blood. To such solutions neutral red and janus green were often added. The many cells that remained adherent to the cover could then be easily studied. Such preparations were usually discarded after study, as they did not remain in good condition for more than a day or so after this treatment. Sometimes a tiny grain of dry neutral red, janus green, or Sudan III was placed with a fine needle on the edge of the culture. The slow diffusion of the dyes into the drop permitted observation of cells with different degrees of staining. The granules, vacuoles, mitochondria and fat globules could be stained in different cells in the same culture when the dye granules were placed some distance apart on the same specimen.

After washing off the thickest part of the drops, fixed preparations were made with iodine vapor or Zenker's solution without the acid.

In some respects the stagnant drop blood-cultures correspond to the extravascular tissue spaces of the subcutaneous tissue and elsewhere, and to the sluggish blood-stream of the spleen. The mononuclears have a better opportunity in such an environment to phagocytize and change into clasmatocytes and epithelioid cells than they have in the swifter blood-stream. The mononuclears may, however, as M. R. Lewis (1925) and others have shown, phagocytize foreign particles while in the blood-stream, then stick to the vessel wall and later migrate out into the tissue spaces as macrophages.

No special effort was made to determine how long the various types of cells lived and no subcultures were made, but the cells often remained alive in cultures for three to four weeks. The observations are based on over 500 blood-cultures from the following animals:

Elasmobranchs:	
Dogfish	4 series
Skate	13 series
Teleosts:	
Sculpin	4 series
Hake	2 series
Cunner	1 series
Flounder	3 series
Fundulus	2 series
Amphibia:	
Toad	5 series
Frog	3 series
Reptiles:	
Garter snake	1 series
Grass snake	1 series
California lizard	1 series

DOGFISH.

Four series of about 20 cultures each were made from dogfish blood taken directly from the heart. Fresh preparations of this blood were especially beautiful, owing to the large size, the great numbers, and the marked activity of the white blood-cells, which were predominantly granulocytes. There were often from 10 to 20 white cells of various sorts in a single field of the 1.5 mm. lens. Occasional pigmented leucocytes were encountered.

The large oval nucleated red blood-cells always contained many small vacuoles, arranged more or less around the centrally placed nucleus. These vacuoles, which stained with neutral red, were found in the erythrocytes, even in blood examined immediately after the cultures were made (fig. 12).

Three types of *granulocytes* with conspicuous granules were noted: (1) Large cells with fairly large oval or round bright granules, often radially arranged about a small clear area, presumably the centrosome (fig. 1); many of these cells were quite motile; (2) large cells, about the same size as the first type, but with pale oval or round granules, likewise more or less radially arranged about a small centrosome (fig. 2); there were transition forms between these two types, so it may be that they are closely related; (3) small cells with small, round, bright granules crowded together (fig. 3). A centrosphere was not observed. In all three types the granules filled the cells except for the centrosome and the nucleus, the latter being at the periphery in the first two types and eccentric in the third.

In the first two types the centrosome occupied approximately the center of the cell and tended to maintain this position as the cells changed form and migrated about. During migration the constant flowing of the granules and changes in the form and position of the nucleus indicated that the centrosome was not definitely fixed in position as regards any particular side of the nucleus. During migration the nucleus was usually at or near the rear end. The granules in all three types reacted to neutral red in varying degrees, those of the small granular type staining most readily. In fixed preparations the granules stained with eosin and resembled eosinophiles. As the granulocytes were followed from day to day they underwent comparatively little change; some were still migrating as late as the fifth and sixth day without any appreciable alteration. Figure 28 shows such a cell stained with neutral red. Some contained a few fat globules and an occasional one contained an ingested red blood-cell or one or two small vacuoles that were stainable with neutral red. There seemed to be an increase in the number of pale granulocytes at the expense of the large bright ones. There was also a fading out of the pale granules in a few of the cells, so that they sometimes became difficult to distinguish from the mononuclears. In the older cultures the granules were somewhat more easily stained with neutral red than they were in the fresh cultures. None of the granulocytes showed much hypertrophy or alteration that would indicate that these cells were becoming transformed into either macrophages or epithelioid cells.

The *lymphocytes* varied greatly in size (figs. 4 to 9). Migration was common and the nucleus was always near the front end of the elongated migrating cell (fig. 5). The lymphocytes always contained a few small granular mitochondria and neutral-red granules, and sometimes a small fat globule or two. The nuclei had the characteristic rather opaque mottled appearance. There was usually a considerable increase in the number of neutral-red granules in these cells during the first 24 hours, and a few of the cells became quite heavily laden with them (figs. 13 and 14). Migrating lymphocytes were observed in cultures up to and including the 6-day ones. The number of lymphocytes seemed to decrease with the age of the culture, but we did not determine whether this was the result of death or of enlargement into forms resembling the mononuclears. The fact that the lymphocytes varied so greatly in size made it possible to arrange them in a graded series, from the smallest ones up to those as large as the mononuclears. This made it difficult to rule out the possibility of the lymphocytes enlarging into mononuclears, which in turn might participate in the formation of macrophages and epithelioid cells.

The *mononuclears* in the fresh cultures were, as already noted, relatively few in number. They varied considerably in character and still more in size; the smaller ones approached in size the large lymphocytes. When flattened out on the cover-glass (fig. 10) they were thin and rather difficult to see. The eccentrically placed nucleus was usually elongated and more or less indented on the inner side. Two nuclei were occasionally present (fig. 11). The nuclei were mottled in appearance and without definite nucleoli. The cells contained a few small granules, some rod-like and granular mitochondria, and sometimes a few fat globules, all of which were often more or less radially arranged about a clear central point, probably the centrosome, which occupied approximately the center of the cell. The thin hyaline-like peripheral layer tended to extend out into thin membranous pseudopodia, or into a thin film-like edge, similar to those of clasmatocytes and epithelioid cells.

Within 24 hours, some of the mononuclears had accumulated fat globules, some exhibited clasmatocyte-like characteristics, and others epithelioid-like ones (figs. 15, 16 and 17). The general appearance of some of these cells, with their film-like pseudopodia, and the way in which they migrated were suggestive of clasmatocytes, even though they contained only an occasional ingested red blood-cell or other material and relatively few neutral-red granules and fat globules. Occasionally, as early as 24 hours, cells were noted with a fairly large central area and an extensive intermediate zone of fat globules (fig. 17); in other words, some had marked epithelioid characters.

During the next few days there was an increase in the number of mononuclears although no division was seen, and all sorts of forms developed, ranging from typical clasmatocyte-like cells (figs. 18, 19 and 20) to typical epithelioid cells (fig. 21), and with numerous intermediate forms (figs. 22 to 25) that were difficult to classify either as clasmatocytes or epithelioid cells. The clasmatocytes contained varying numbers of irregularly arranged ingested red blood-cells and cellular debris undergoing digestion, and varying numbers of fat globules irregularly scattered in the cytoplasm. Owing to the mass of ingested material, the centrosome was usually obscured and often the cell had to be followed for some time before the nucleus could be distinguished. The freshly ingested red cells were smaller than the normal and frequently of a deeper hæmoglobin color. They seemed to undergo a process of dehydration during ingestion. The larger ingested red cells did not as a rule stain with neutral red, nor were they surrounded by vacuoles. As digestion proceeded they sometimes became laked, in other instances crumpled. Within a few hours after ingestion they had become much smaller and were then usually stainable with neutral red and sometimes surrounded by vacuoles, the fluid contents of which were also stainable with neutral red. It was often difficult to determine whether a partly digested red cell was laked or was enclosed in a vacuole; many of them seemed to be undergoing digestion without any accompanying vacuole. There was no indication that the ingested reds were ever taken into pre-existing vacuoles. All the larger ones and many of the smaller ones were free in the cytoplasm without any perceptible vacuole surrounding them. After a few days, some cells contained ingested erythrocytes (figs. 19 and 20) in various stages of digestion, others only small remains of the ingested cells, and others indefinite material which stained with neutral red (fig. 18). In the older cultures there were comparatively few of the typical epithelioid cells like the one shown in figure 21, with a large central area of fine neutral-red granules surrounded by a zone of fat globules. This cell contained an unusually large clear centrosphere. The majority of the somewhat hypertrophied and altered cells were intermediate in type, as shown in figures 22 and 25. The nuclei of the macrophages and epithelioid cells enlarge parallel with the increase in the size of the cells, lose their mottled appearance, become more homogeneous and transparent and usually a definite nucleolus can be seen. Those of the giant cells are much smaller but otherwise show the same changes.

A few cultures were observed as late as the tenth day. The epithelioid giant cell from such a culture, shown in figure 26, had a finely granular central area containing a few brown pigment granules surrounded by an intermediate zone containing fat globules. There were 3 nuclei and the remains of ingested cells. The peripheral layer of the cell extended into thin film pseudopodia. Occasionally the enlarged cells became stretched into spindle forms (fig. 27).

After a few days many of the cells that had been attached to the cover-glass dropped off into the depths of the fluid drop and could not be followed. Smears of such fluid drops sometimes contained a few giant cells.

SKATE.

Thirteen series of from 10 to 20 cultures each were made from blood taken directly from the heart of four species of skates. Most of the cultures were from *Raia erinacea*. As the white cells from the other species behaved in much the same manner, we shall not consider them separately.

The blood-cells of the skate, like those of the dogfish, are considerably larger than similar cells of the bony fishes examined. The conspicuous *granulocytes* were numerous in the fresh cultures and were frequently migrating. When these cells became spread out more or less on the coverslip, a small, distinct, clear centrosome could be seen surrounded by granules, often radially arranged. The granules varied from spindle-shaped to round forms and had the usual affinity for neutral red (figs. 29, 30, 31). The eccentrically placed nuclei varied from oval to lobulated forms, and cells with 2 nuclei were occasionally seen. During migration the granular end was in front and the nuclear end at the rear. As the cells changed form and twisted about in their migration, one could see that the centrosome, as in similar cells from the dogfish, bore no fixed relation to any particular side of the nucleus. As the cells moved the centrosome was often more or less obscured, but it tended to maintain a central location, separated from the nucleus by 1 to 6 layers of granules. The granules were often in motion, partly Brownian and partly cytoplasmic. The size of the granules varied in different cells, and sometimes even in the same cell. The cells with the larger granules seemed to show more migration as well as more movement of the granules themselves. The shape of the granules varied;

some cells contained only spindle forms, others only round ones, and others had both forms. The mitochondria, difficult to recognize without janus green, were granular, relatively few in number, and scattered near the periphery of the cell and in the region of the nucleus. Some cells seemed to contain very few, but this may only have been because of the difficulty of recognizing them.

The *mononuclears* (figs. 32 to 36) varied greatly in the fresh cultures, in number and in size. Some were fully as large as the granulocytes, others considerably smaller; some had an oval nucleus and a radial arrangement of fine granules and mitochondria about a centrosome. Others did not show such a radial arrangement, the granules being more or less scattered about in the cytoplasm. Some of the cells had, in addition to granules, varying numbers of vacuoles with an affinity for neutral red and sometimes a few small fat globules. The small granular and rod-shaped mitochondria varied in number and in arrangement (figs. 35, 36).

The *lymphocytes* were numerous, some small, others nearly as large as the mononuclears (fig. 37). It was often difficult to decide whether to classify a cell as a large lymphocyte or as a mononuclear. When the lymphocytes were examined with a little neutral red and janus green immediately after the cultures were made, they were often entirely without neutral-red granules. In the course of a few hours, however, such granules almost always appeared and frequently increased in number during the next day or two (fig. 51). The mitochondria were small and relatively few in number, 15 to 25 having been observed, and were more or less clumped at one side of the nucleus.

The *erythrocytes* (figs. 38, 39, 50) were nucleated and showed considerable variation in size, ranging from large oval ones to small round ones. Some contained many small vacuoles with an affinity for neutral red, while others had only a few. Scattered short-rod mitochondria were observed in them after staining with janus green (fig. 39).

After 24 hours some of the cultures showed very little change in the character of the various types of cells, while others had a marked increase in the number of the mononuclears which had begun to accumulate fat globules and neutral-red staining bodies. There was considerable variation in the number and size of the fat globules and in the number and size of the neutral-red bodies. Some of the cells had become clasmatocyte-like and contained ingested red blood-cells. The majority of the granulocytes, on the other hand, were little changed, except for the presence of a few fat globules. At the extreme periphery of the drop some of these cells appeared to be losing their granules, which often seemed to dissolve away, until in some cells there was present only a radial granulation immediately about the centrosome.

Most of the 48-hour cultures showed little change in the granulocytes. There were more neutral-red granules present in the lymphocytes (fig. 51). The mononuclears had increased in numbers and showed a great variation in the number of fat globules and neutral-red bodies. Some of these cells had a decidedly clasmatocyte-like appearance but had not yet become markedly increased in size (figs. 52, 53, 54, 55 and 56).

During the next few days the mononuclears increased in size and more of them became transformed into macrophages, but the other types of cells remained about the same. Some cultures that were followed for 10 or 12 days still contained numerous migrating granulocytes with a few fat globules (figs. 40, 41) and a few small lymphocytes. Most of the mononuclears contained large numbers of neutral-red bodies consisting of all sorts of debris, masses of pigment granules, and red blood-cells undergoing digestion (figs. 42 to 47). Some of the ingested red blood-cells, apparently freshly ingested, contained hæmoglobin (figs. 48 and 49), while others were pale as though laked. The ingested red blood-cells, even when they contained hæmoglobin, were always considerably smaller than those that were free in the drop. (Compare the ingested red cells in figures 48 and 49 with the normal size in figures 38, 39, and 50.) Some of the ingested debris appeared to be from dead leucocytes. The number and size of the fat globules varied considerably. The hypertrophied cells (figs. 42 to 49) are typical macrophages. Typical epithelioid cells were not seen but the cells shown in figures 43 and 47 approach somewhat the epithelioid type.

Nuclear changes similar to those observed in the mononuclears of the dogfish occurred in those of the skate, namely, hypertrophy, clearing and the development of definite nucleoli (figs. 42, 43).

SCULPIN.

In the fresh hanging drops of the sculpin's blood the *granulocytes* were for the most part spherical (fig. 57) and scarcely as large as the red blood-cells. They were crowded full of highly refractive granules with a marked affinity for neutral red. A few of them were usually migrating about. No particular changes were observed in these cells during the 7 days in which the cultures were followed, except the swelling of some of the granules.

The *lymphocytes* (figs. 58 to 63) were abundant, migrated actively and varied considerably in size and contained from 2 to 13 neutral-red granules. Some of the larger cells, with more than the average number of granules, approached the mononuclears in character and suggested the possibility of the transition of one type of cell into the other.

The *red blood-cells* (figs. 70, 71) varied considerably in size and often contained from one to three small neutral-red granules in fresh cultures and sometimes a few more in the older ones.

The *mononuclears* (figs. 64 to 69) were more numerous than the granulocytes and often were abundant about the periphery of the drop, toward which they frequently migrated. They usually contained many small neutral-red granules. They readily ingested red blood-cells and sometimes within an hour or two some contained one or two red blood-cells or other ingested materials. These cells seemed to become transformed more rapidly than those in the other types of blood, and frequently at the end of 24 hours many were already hypertrophied and contained fat globules as well as ingested red blood-cells.

In the 48-hour cultures astonishing hypertrophy and transformation had already taken place. Epithelioid cells (figs. 72 to 74), macrophages (fig. 75), and even giant cells (fig. 76) were often fairly abundant. A characteristic epithelioid cell is shown in figure 72. It is several times larger than the normal mononuclear and contains the characteristic, very finely granular, almost clear central area with an affinity for neutral red (not stained in this figure). About the central area is the characteristic intermediate zone of fat globules in which lies the large nucleus. The clear peripheral cytoplasm is spread out into a thin sheet. The nucleus is much hypertrophied, the nucleoplasm is clear, and a distinct nucleolus is present. Many of the transformed cells contained numerous spindle or needle-like bodies of uncertain origin, which resembled crystals. The macrophages (fig. 75) were likewise very much larger than the mononuclears of the fresh cultures. They were more or less amœboid and contained numbers of large ingested masses, especially red blood-cells and irregularly distributed fat globules. The close relationship or identity of the epithelioid cell and macrophage is clearly shown in figures 72 to 75. While the cell (fig. 73) was under observation the broad, thin, sheet-like peripheral layer contracted toward the body of the cell and it assumed the form shown in figure 74. Half an hour later it again became spread out somewhat as it was in figure 73. Were the freshly ingested red blood-cells absent it would undoubtedly be typically epithelioid-like. The epithelioid giant cell shown in figure 76 contains the usual clear central area, the intermediate zone of fat globules in which are imbedded 3 nuclei, and the clear sheet-like peripheral layer. A couple of ingested red blood-cells and numerous needle-like bodies are present.

The 7 and 8-day cultures contained numerous epithelioid cells and macrophages. The elongated epithelioid cell shown in figure 77, from a culture stained with neutral red, contains a finely granular central area composed of neutral-red granules, an intermediate zone of many fat globules, partly digested red cells, and other debris, and a single large nucleus. The elongated one shown in figure 78 did not contain ingested masses. Its central area and the intermediate zone of fat globules are evident. The typical macrophages from a 7-day culture (figs. 79, 80, 81) had many irregularly distributed neutral-red bodies, varying numbers of ingested red blood-cells, and scattered fat globules. Most of the neutral-red bodies were probably the remains of digested red blood-cells. The freshly ingested ones, although considerably reduced in size, did not take the neutral-red dye.

As with the dogfish and the skate, we never found ingested red blood-cells as large as the free ones. The ingested red blood-cells shown in figures 77, 79, 80, and 81 are considerably smaller than those shown in figure 73. The cells shown in figures 79, 80, and 81 are all from a culture that had been stained 2 days previously with neutral red. The condition of the ingested material in these cells suggests that a considerable interval had elapsed between the time the last of the bodies stained with neutral red were ingested and the time when the unstained red cells were ingested. It also suggests that the stainability of ingested material with neutral red may depend upon the penetration and partial digestion of the same by the digestive ferments or juices.

HAKE.

The cultures of blood from the hake (one of the bony fishes) were followed for only 5 days. The granulocytes and lymphocytes were not numerous and underwent very little change. The mononuclears were numerous in the fresh cultures and usually contained a few neutral-red staining granules and small vacuoles near the nucleus, which was relatively large. During the period of observation these cells were extremely phagocytic. They ingested varying numbers of red blood-cells and other smaller masses, accumulated large numbers of fat globules and became considerably hypertrophied. Some contained red blood-cells in various stages of digestion and numerous fat globules more or less scattered between the ingested material. Such cells with irregular, thin membranous pseudopodia were of the macrophage type. Some of the other mononuclears contained only granules that were more or less concentrated into a central area at the side of the nucleus. About this area many fat globules formed in an intermediate zone. These epithelioid cells were not as typical as those in some of the other forms. Intermediate types were also abundant. No giant cells were observed.

CUNNER.

Only one series of cultures was made from the cunner. In the fresh cultures many of the white blood-cells were actively motile. Granular leucocytes (fig. 86), lymphocytes (fig. 85), and mononuclears (fig. 87) were present. The mononuclears were about half the size of the red blood-cells (fig. 88) and about the same size and as numerous as the granulocytes. In the cultures they hypertrophied and became transformed as in the other bony fishes described. After 6 days many of them had become changed into large epithelioid cells, 5 or 6 times as large as a red blood-cell. They contained a central granular area surrounded by a zone of rather coarse fat globules. The oval or bean-shaped nucleus eccentrically placed at one side of the central area usually interrupted the zone of fat globules. A few of these cells contained an ingested red blood-cell (figs. 82 and 83). Typical clasmatocytes were relatively few. Giant cells (figs. 84, 89) with 2 to 20 nuclei were observed. Some typical granulocytes and lymphocytes were still present in the cultures after 6 days.

TOAD.

The blood-cells in fresh cultures of the toad were considerably larger than those found in the bony fishes. Typical polymorphonuclear leucocytes (figs. 97, 98) were numerous and usually active. The granulocytes (fig. 90) were the most numerous of the while blood-cells. The mononuclears (figs. 91, 92) varied considerably in size, but none was quite as large as the red blood-cells (fig. 93). Many were migrating and undergoing changes of form. They often contained neutral-red staining bodies and fine fat globules. The lymphocytes presented the usual characteristics and many of them were migrating about (figs. 94, 95, 96).

The cells in the 24-hour cultures were very little changed. In the 48-hour cultures some of the polymorphonuclear leucocytes had developed pseudopodia, more like those of the clasmatocytes than the ones previouly seen on these cells. The eosinophiles, still the most numerous type, were almost unchanged. Most of them were round, and only rarely was a migrating form seen. The lymphocytes, too, were very little changed and comparatively few were encountered. Here and there were found patches of from 20 to 50 thin, flat cells, thrombocytes (fig. 108) with large round nuclei, fine granules, and large, thin pseudopodia. Many were somewhat motile.

In the 3-day cultures there was a noticeable enlargement of the mononuclears, some being several times as large as the red blood-cells. The majority of these enlarged cells were of the epithelioid type or were undergoing transitions toward the epithelioid type (figs. 99, 100, 101); the larger ones contained the typical central area, full of fine granules with an affinity for neutral red, surrounded by a zone of fat globules (fig. 111). The oval nucleus was at one side of the central area. Clasmatocyte-like forms (fig. 102) and forms intermediate between these and the epithelioid were fairly common.

On the fourth and fifth days the number of epithelioid and clasmatocyte-like types (figs. 103, 104, 105, 106, 107) had increased and a number of giant cells of the epithelioid type had developed. Both types of cells, but more especially the clasmatocyte-like type, contained, on the third day and thereafter, varying numbers of red blood-cells in various stages of digestion. During the second week there were many epithelioid cells (fig. 116), macrophages, and giant cells of the epithelioid type (fig. 115). Even after 2 weeks the cultures contained clasmatocytes full of ingested red blood-cells, some of which were freshly ingested and still retained the hæmoglobin, many epithelioid cells and some giant cells (figs. 112, 113, 114). Many of the giant cells (too large to be figured) were especially beautiful; some contained 40 to 50 nuclei arranged around the characteristic central area (figs. 115, 116). In addition to the giant cells of the Langhans type there were often very large plasmodial-like giant cells covering large areas and containing many nuclei that were very irregularly distributed. In some parts of the plasmodial mass the nuclei were scattered, in other parts they were concentrated in large groups. While the giant cells of the Langhans type show no indication of formation by fusion, the plasmodial ones often appear as though they were formed, in part at least, in this manner. It thus seems not unlikely that in the toad and frog giant cells arise both by division of the nucleus without division of the cytoplasm and by fusion of cells. It would seem an easy matter to follow this in the living, but in spite of many attempts we have not yet succeeded.

In most cultures there was an enormous increase in the number of thrombocytes or hæmoblasts (figs. 108, 109, 110). They were partly massed in dense clusters, partly spread out in thin sheets, and partly scattered. These small cells remained fairly constant in appearance during the life of the cultures. They varied in shape in different parts of the culture from thin flat disks to slender spindle forms according, probably, to physical conditions of their immediate environment. The cytoplasm is clear and thin and when spread out on the cover-glass difficult to see in the living state. They have a small mitochondrial content. Very rarely do they contain a small ingested mass. The larger ones approach somewhat in size the smallest mononuclears, but there is no obvious transformation into the latter.

LEOPARD FROG.

The changes which take place in the frog's blood have been described more fully elsewhere (M. R. Lewis, 1926). The mononuclears behaved in the usual manner, exhibiting phagocytosis, hypertrophy and multiplication, and in the course of a few days many were changed into epithelioid cells, clasmatocytes, and giant cells (figs. 117 to 129).

In cultures of blood from both the toad and the frog we were able to follow sometimes the changes produced in the appearance of such macrophages as shown in figures 102, 104 and 126, as the large ingested red cells were reduced to granules by digestion. Such reduction in the size of the granules produced epithelioid cells much like those shown in figures 99, 100, and 101. Most of the epithelioid cells, however, seemed to be formed by the ingestion or accumulation of small granules, probably fibrin or plasma. It is significant that cells which show no apparent central area develop or reveal a very prominent one when the larger ingested bodies are reduced in size. There are many indications that most of the macrophages contain a central area that is more or less obscured by the larger masses. Typical epithelioid cells can be changed into macrophages if the cultures are turned upside down so that the red blood-cells fall on them. Ingestion of large numbers may then take place with a resulting marked change in the character of the epithelioid cell, which becomes macrophagic in appearance.

GARTER SNAKE.

In the fresh cultures of the blood from the garter snake there were two types of granulocytes. The larger ones (fig. 130), about two-thirds the size of the red blood-cell, were crowded with bright granules, which, under favorable conditions, showed a definite radiation from a very small central area. The smaller forms were crowded with hazy granules (fig. 131). The mononuclears (fig. 132) were smaller and often motile, and sometimes contained a few scattered fat globules. The lymphocytes (fig. 133) were rather scarce, often migrated, and had the usual characteristics. Some polymorphonuclear cells were present. The white blood-cells in a field were relatively few as compared with some of the other forms studied. The nucleated red blood-cells (fig. 134) were usually without vacuoles.

In the 24-hour cultures the lymphocytes and granulocytes showed no especial change. The mononuclears, relatively few in number, were somewhat enlarged and were undergoing rapid change of form and more or less migration by means of thin membranous pseudopodia. They contained relatively few vacuoles, fat globules, and ingested stuffs, and were rather difficult to see. No ingested red blood-cells were observed, either in the 24 or 48-hour cultures. In the latter cultures some of the mononuclears, containing a few vacuoles and small granules, were quite clasmatocyte-like in appearance and behavior.

The mononuclears hypertrophied and slowly changed. On the seventh day there were epithelioid-like (figs. 135 to 137) and clasmatocyte-like forms. Occasionally ingested red blood-cells were present as in the epithelioid cell (fig. 135). It will be noted that the ingested red blood-cell in this hypertrophied mononuclear lies against the nucleus, more or less obscuring the centrosome area. The cells continued to grow slowly and on the thirteenth day the epithelioid type (figs. 138, 139) was fairly characteristic, except that the accumulation of fat globules was scanty. The cell shown in figure 139 contained almost none. The clasmatocyte-like cells shown in figures 140 and 141 likewise contained very few fat globules. It is quite evident that the cells shown in figures 135 to 140 are all merely modified mononuclears and that the differences in appearance are due to the amount, character and degree of digestion of the ingested material and upon the extent of the hypertrophy.

Cultures observed on the twenty-first day contained living granulocytes, which were but little changed, and many phagocytic cells. The cultures were still alive on the twenty-sixth day and contained many large epithelioid cells and many other large phagocytic cells which varied in character from the epithelioid to the clasmatocyte types.

GREEN GRASS SNAKE.

The fresh cultures of the blood resembled closely those from the garter snake. No especial changes were noted in the 24-hour cultures. The mononuclears of the 48-hour cultures were considerably enlarged and often contained a few fat globules and irregular debris.

By the third day many of the mononuclears had markedly increased in size and accumulated neutral-red staining bodies and fat globules. Occasionally, ingested red blood-cells were seen (fig. 144). When such cells became spread out on the cover-glass it could easily be seen that some were of the epithelioid type, containing fat globules and a central area with granules having an affinity for neutral red (fig. 142). In some of the cells small vacuoles were scattered among the fat globules at one side of the nucleus, or about a central area (figs. 143, 144, 145). Others, clasmatocyte-like in appearance, showed more irregular distribution of the vacuoles and fat globules (fig. 146) and no indication of a central area. The eosinophiles showed no especial change (figs. 147, 148). The red blood-cells sometimes contained one or two small neutral-red staining granules (fig. 149).

CALIFORNIA LIZARD.

The white blood-cells in the fresh preparations of lizard's blood were quite numerous. Two types of granulocytes were observed; one with round and the other with spindle-shaped granules. Many of the latter were binucleated and migrated actively about (figs. 150, 151). The ones with round granules (figs. 152, 153) were not nearly as active and seemed to be dying. The lymphocytes (figs. 154, 155, 156) and the thrombocytes which were abundant, together with granulocytes, constituted the bulk of the white cells. The mononuclears (figs. 157, 158, 159) were relatively few in number. Some had a distinct radial arrangement of the mitochondria about a point at one side of the nucleus (fig. 159). Rarely cells contained a few fat globules arranged about a rather large central area (fig. 158). The white blood-cells were smaller than the red (fig. 160).

The mononuclears hypertrophied, increased in number, and changed rather slowly, but on the sixth day cells of the epithelioid type (figs. 161 to 163) and of the macrophage type (figs. 164, 165) were already present. The latter contained numbers of ingested red blood-cells and often some fat globules that were irregularly distributed in the cytoplasm. The ingested red cells were always considerably smaller than the free ones. Some retained their hæmoglobin while others were laked. Ultimately, however, they all lost their color. The large, freshly ingested red cells were not surrounded by vacuoles, nor did they stain with neutral red. There was no indication that they were ever taken into pre-existing vacuoles. Later, as they were being digested, they stained with neutral red. Sometimes vacuoles developed about them, and these together with the enclosed red cell stained with neutral red. The macrophages often contained such quantities of ingested material that the central area, and even the nucleus, was obscured. Some of the hypertrophied cells, especially those flattened out on the cover-glass, had the typical epithelioid characters (figs. 161, 162). Figure 162 shows this best. The large finely granular central area is surrounded by an intermediate zone of fat globules. Beyond this a zone of thin clear cytoplasm extends into thin membranous pseudopodia or a migrating edge. The cell shown in figure 161 had only a partially developed intermediate zone of fat globules. Sometimes this typical arrangement of central area and intermediate zone of fat globules was lost, temporarily at least, as the cells moved about (fig. 163). When such cells rounded up and became spherical, the arrangement of central area and intermediate zone corresponded more to the usual picture of the living epithelioid cells in spreads from tuberculous lesions. The nuclei of these hypertrophied cells, eccentrically located in the intermediate zone, were considerably enlarged and contained a clearer nucleoplasm and more definite nucleoli than the mononuclears. The granulocytes (fig. 166), lymphocytes (fig. 167), and hæmoblasts were not apparently altered at this time. The 8 and 10-day cultures contained an increased number of hypertrophied cells, macrophages (figs. 168, 169), epithelioid cells (fig. 170), and occasional giant cells (fig. 171). Some of the hypertrophied cells, probably epithelioid, were stretched into more or less spindle-shaped forms (figs. 172, 177). The amount of hypertrophy which had taken place can be estimated roughly by comparing these figures with those of the mononuclears in the fresh cultures. When such cells were stained with neutral red (figs. 178 to 180) the granular central area was revealed to great advantage. The central area in some cells (fig. 179) seemed to consist more of vacuoles than of granules, and there was more intermingling of the fat globules and vacuoles than in the typical epithelioid cell. The elongated cell shown in figure 181 had more of the macrophage than of the epithelioid character, as can be seen by the irregular distribution of the large neutral-red bodies and fat gloubles. Even in the 8-day cultures there were still present granulocytes and lymphocytes (figs. 173, 174) that were very little changed. Some cells were seen that offered difficulties in classification (fig. 175).

The cultures were examined at varying intervals during the next 3 weeks. There seemed to be, for a time at least, an increase in the number of transformed cells, as well as an increase in the variety of forms and variations in structure. In some cultures were found patches of transformed cells containing macrophages, epithelioid cells, and giant cells (figs. 182, 183). Although there was often more or less overlapping of such cells, there was no particular evidence of any fusion. In some places, as shown in figure 182, all three types were often lying side by side in approximately the same environment. Some of the transformed cells assumed somewhat the appearance of connective-tissue cells (fig. 185), and intermediate stages (figs. 184, 187, 188) between them and the typical epithelioid cell shown in figure 184 were encountered. Stretched-out cells were occasionally seen to round up again into an epithelioid form. Migrating cells in fairly good condition were observed on the seventeenth day (fig. 189).

The last culture of the series, examined on the thirty-fourth day, still contained a great variety of living cells that were full of granules having a marked affinity for neutral red (figs. 186, 190). Round cells, flat cells, spindle cells, and multipolar cells were all fairly abundant. Many remarkable forms were observed, but the general cytological characters were obscured by the great accumulation of granules and vacuoles, due to degenerative changes.

Thrombocytes (fig. 191) were very abundant in the cultures. They contained small mitochondria. Some of them had a peculiar oval zone a short distance from the nucleus; between it and the nucleus were the mitochondria. We have not investigated the meaning of this but it suggests a transition stage toward the red blood-cell.

SUMMARY AND DISCUSSION.

From the preceding observations it is apparent—

(1) That the mononuclears, macrophages and epithelioid cells are merely different phases of the same cell type.

(2) That the transformation of the mononuclears into macrophages, epithelioid cells, and transition forms can scarcely be considered as a true differentiation into new and fixed types of cells.

(3) That these different forms are more in the nature of temporary functional variations of the same cell type.

(4) That the differences in their appearance depend to a great extent upon the amount and character of the ingested material and upon the degree of digestion and assimilation of the same. Thus, the mononuclears that accumulate numbers of large masses, such as red blood-cells, become clasmatocytes, while those that accumulate large numbers of small granules become epithelioid cells.

(5) That clasmatocytes can take on the epithelioid characters as the ingested materials are reduced to fine granules by digestion.

(6) That epithelioid cells can, under certain conditions, ingest many red blood-cells and become clasmatocytes.

(7) That the numerous transition forms between the clasmatocytes and epithelioid cells are in keeping with our findings concerning the relationship existing between them.

(8) That the hypertrophy of the cells depends to a great extent upon the excessive amount of ingested material and its synthesis into protoplasm.

(9) That the similarity between the macrophages derived from the mononuclears in the blood-cultures and those found so abundantly scattered through the body seems to prove beyond question that the macrophages from the two sources are identical, and this leads to the logical assumption that the macrophages of the body are derived either directly or indirectly from the mononuclears of the blood.

(10) That the similarity between the epithelioid cells derived from the mononuclears in blood-cultures and those found in tuberculous lesions seems to prove beyond question that the epithelioid cells from the two sources are identical, and this leads to the logical assumption that the epithelioid cells of tuberculous lesions are derived either directly or indirectly from the mononuclears of the blood.

The great divergence of opinion regarding the origin in the adult of the macrophages and epithelioid cells can be ascribed to the lack of decisive methods of investigation. The connective tissue, endothelial, epithelial, and leucocytic theories of origin for these cells have each distinguished and energetic supporters, who have based their ideas for the most part on the interpretation of fixed, sectioned and stained material. This method has very decided limitations and can rarely be final for such problems, as actual transformation can not be observed in dead material. The arranging of supposed transition forms into a definite series will always be an interpretation, and as such is liable to error. In addition to this difficulty, the picture, as presented by the usual techniques, is more or less confused by the fact that in the body various types of cells, such as connective tissue, endothelium, and blood-cells, are present in the same field, and the intricate changes taking place under such conditions are usually too difficult for the eye to resolve, either in the living or in a series of fixed and sectioned preparations.

The claim for the exclusive leucocytic origin of the macrophages dates back to the time of Cohnheim, Koch, and Metschnikoff. Since then, numerous investigators have claimed that these cells develop, in part at least, either from the lymphocytes or the mononuclears (endothelial leucocytes).

The idea that the white blood-cells are directly related to one another and to the macrophages, the epithelioid cells and the giant cells of the Langhans type has recently received support by Maximow (1924) in a study in which the tissue-culture technique was employed. Under suitable conditions he claims that the "reticular cells" of the lymph-node hypertrophy, divide, and become enlarged into polyblasts (macrophages) and epithelioid cells; and even the lymphocytes themselves, he claims, may undergo similar transformations. Unfortunately Maximow's observations are, to a great extent, based on fixed, sectioned, and stained cultures of such tissues as lymph nodes, omentum, and connective tissue, where connective tissue, endothelium and leucocytes are present. He passes over his direct observations on the living cells in so few words that they hardly offer more convincing proof in support of his views than do his observations from the fixed, sectioned and stained cultures, and the latter present much the same difficulties as do observations on material from the body.

The earlier observations of Awrorow and Timofejewskij (1914) on cultures of leukæmic blood, where the myeloblasts and lymphocytes were found by them to hypertrophy and form macrophages, giant cells, and spindle or multipolar cells resembling fibroblasts, are in accord, in part at least, with the findings of Maximow and in part with those of Carrel and Ebeling (1922), for they too obtained macrophages and fibroblast-like cells in cultures of hen's blood. According to them, the lymphocytes and eosinophiles die or disappear, leaving pure cultures of mononuclears from which the macrophages, and especially the fibroblast-like cells, arise. Multipolar and spindle shaped fibroblast-like cells, similar to those found by Carrel and Ebeling and presumably by Awrorow and Timofejewskij, have been encountered by us in our blood-cultures. Although some resembled fibroblasts, a more prolonged study of their cytological characteristics and general behavior indicated that they were merely elongated or multipolar shapes temporarily assumed by macrophages and epithelioid cells, since some of them may later change to the more characteristic forms. These cells occurred in great profusion in cultures of lizard's blood.

Awrorow and Timofejewskij and Maximow claim that the lymphocytes can become transformed into macrophages. Carrell and Ebeling, on the other hand, found that the lymphocytes disappeared and that only the mononuclears were transformed. In our cultures there were always some lymphocytes present, and while our observations show that the mononuclears are transformed into macrophages, they still leave us in some doubt as to the fate of the lymphocytes. The difficulties of following the changes which take place in individual cells are considerable, as they must be kept almost constantly under observation for several days. The changes which the mononuclears underwent were so obvious, however, that it was scarcely necessary to follow them from hour to hour, as most cultures after a few days contained all stages of their transformation into macrophages. No such obvious transformation of lymphocytes were encountered by us and, although there were some indications of it, they were not convincing enough to enable us to settle the question without more complete data, which can only be obtained by following individual lymphocytes from hour to hour and day to day.

According to the present views of the school represented by Mallory (1898), Permar (1924), and Foot (1925), under the term endothelial cell or endothelial leucocyte are included those cells variously described in the literature as the (large) mononuclear cell, macrophage, clasmatocyte, histiocyte, resting wandering cell, wandering connective-tissue cell, adventitial cell, pyrrhol cell, cellule rhagiocrine, etc. The dust cells of the lung and the epithelioid cells of tuberculous lesions are also included. These investigators believe that these cells are all derived from the capillary endothelium. Our observations are in agreement with the idea that the mononuclears, the clasmatocytes, and the epithelioid cells are merely modifications of the same cell type. Naturally our blood-cultures do not give us any clue as to the origin of the mononuclears from the endothelium. Maximow's observations and those of Mallory and Foot and Permar seem to agree as regards the close relationship of mononuclears, clasmatocytes, and epithelioid cells. They differ, however, as regards their origin, since Mallory and Foot and Permar believe these cells come from the endothelium, while Maximow derives them from the lymphocytes and "reticular cells."

In marked contrast to these conclusions are the recent ones of Sabin, Doan, and Cunningham (1925 *a, b, c*)[1] to the effect that the clasmatocytes and monocytes (mononuclears) belong to two very distinct types of cells as regards derivation, cytological characteristics, and function. They claim that the clasmatocytes are derived from endothelial cells and the monocytes from "reticular cells." Their evidence of a different origin for the two cells rests, in the first place, upon the observations of Sabin (1920, 1921) on the living blastoderm of the chick in hanging-drop preparations. She first claimed that there both types of cells came from the endothelium, the clasmatocytes on the outside and the monocytes on the inside. Later, with Doan and Cunningham (1925*a*, p. 134) she concluded that the cells which came off from the inside of the endothelial wall were also clasmatocytes because subsequent observations indicated "that the clasmatocyte is not identical with the monocyte, but rather that they are two types of cells from the standpoint of origin and function." This new evidence for the separation of the clasmatocytes from the monocytes (mononuclears) rests on the study of living cells from splenic punctures with vital dyes and on the study of the origin of the white blood-cells from a primitive "reticular" cell of regenerating bone marrow. These authors (1925*c*) believe that by this method they have determined that the monocyte comes from a primitive cell wholly similar to that from which the lymphocyte and polymorphonuclear leucocyte are derived.

The fact that the clasmatocytes and the mononuclears, in their preparations, present strikingly different cytological characters does not necessarily preclude the possibility of the transformation of one form into the other or of a common origin. There is, however, good evidence against the separation of clasmatocytes and monocytes into two types on the basis of cytological differences. In the first place, these authors claim that the clasmatocytes may be separated from the monocytes on the basis of their mitochondrial content. They claim (1925*a*, p. 136) that large clasmatocytes may occasionally contain a few mitochondria but that the vast majority of the large ones contain none at all. We have been unable to confirm this, and have observed mitochondria in practically all the clasmatocytes studied in spreads and in cultures where janus green alone was employed. When macrophages, heavily laden with ingested material, are deeply stained with a combination of neutral red and janus green the mitochondria are sometimes difficult or impossible to see, as the red color obscures them.

In the hanging drops of blood the mononuclears that have ingested many red cells and have come to resemble large clasmatocytes sometimes fail to show mitochondria, even with janus green, not because the latter are absent but because the cells are so large that the dye either does not penetrate or the mass of ingested stuff obscures them. Spreads of the deep subcutaneous fascia of the rat contain great numbers of large clasmatocytes and when these are properly stained with janus green they reveal many short rod-shaped and granular mitochondria. The mitochondrial content, here at least, seems to increase with the size of the cell. The supposed absence of mitochondria in the clasmatocyte is then not a distinguishing characteristic.

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[1] The three joint papers of these authors will be referred to as follows: Sabin, Doan, and Cunningham = 1925*a*; Doan, Cunningham, and Sabin = 1925*b*,; Cunningham, Sabin and Doan = 1925*c*.

The presence of a centrosome and its granular rosette in the monocyte and its absence in the clasmatocyte has also been used by these authors as a criterion for separating clasmatocytes from mononuclears. We have often seen, however, a central area or centrosome in typical clasmatocytes, sometimes in ordinary spreads, more often in cultures where the cells were flattened out on the cover-glass. Under ordinary conditions, as in spreads where the cells are not flattened, the presence of large numbers of granules and vacuoles, so characteristic of the clasmatocytes, obscures this body and, where vacuolization is very extensive, reduces it in size. As pointed out by Dr. Sabin, the monocytes (mononuclears) contain an unusually easily distinguishable centrosome, but this body often becomes obscured after the mononuclear has ingested large numbers of red blood-cells. It is probably still present when impossible to see. The visibility and size of a centrosphere is a variable matter in other types of cells, such as endothelium and mesenchyme, and depends upon the condition of the cell (W. H. Lewis, 1920).

There is also no good evidence for a separation of these cells into two types from the standpoint of function, as Sabin, Cunningham and Doan suggest (1925*a*, p. 134), for even they state (p. 129) that "phagocytosis is a very general property of cells, but certain cells, such as clasmatocytes and monocytes, have this property accentuated for a physiological purpose of the organism as a whole." Thus, on cytological and physiological grounds, there is no reason for separating the clasmatocytes from the mononuclear macrophages. Regarding their separation on the basis of difference in origin we might add that the question of origin of these cells is by no means settled and we need to know much more before there can be any general agreement.

There are thus at the present time in this country at least three schools of thought as regards the cells (mononuclears, clasmatocytes, and epithelioid cells) with which we are especially concerned. The first, represented by Maximow, derives these three cell types either directly or indirectly from the lymphocytes and "reticulum" cells. The second, represented by Mallory, Foot, McJunkin, and Permar, derives them, either directly or indirectly, from capillary endothelium; and the third, represented by Sabin, Cunningham and Doan, derive the clasmatocytes from the endothelium and the mononuclears from a primitive reticular cell. Our observations agree with those of the first two schools in bringing together under one cell type the mononuclears, clasmatocytes and epithelioid cells, and differ from those of the third school regarding any fundamental distinction between the mononuclears on the one hand and the clasmatocytes on the other.

CONCLUSIONS.

1. Many of the mononuclears in hanging-drop cultures of the blood become transformed into clasmatocytes or macrophages, into epithelioid cells, and into giant cells of the Langhans type.

2. The mononuclears of the blood, the clasmatocytes, and the epithelioid cells are merely different forms of the same cell type.

3. The differences between them depend to a great extent upon the amount and character of the ingested material and upon the degree of digestion and assimilation of the same.

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NOTE REGARDING ILLUSTRATIONS.

The outlines and as much of the detail as possible of figures 1 to 181 were done with the camera lucida, and all except 108, 109 and 110 were drawn from living cells. Some details were filled in free hand while the specimen was under observation. The magnification of all the drawings is about 1,500 diameters, except figures 115 and 116, which are 750 diameters.

Figures 182 to 191, are photographs from cultures that had been washed with a modified Locke solution with or without neutral red or janus green and fixed with iodine vapor. All the cells that remain after washing are those that are attached to the cover-glass. The nuclei in figure 188 were retouched.

PLATE 1.

DOGFISH.

FIG. 1. Eosinophile with bright granules; from fresh blood.

FIG. 2. Eosinophile with pale granules; fresh blood.

FIG. 3. Small eosinophile with round granules; fresh blood.

FIGS. 4 to 9. Lymphocytes from fresh blood; 4 and 5, small lymphocytes; 5 migrating; 6, 7, 8, and 9, large lymphocytes.

FIGS. 10, 11. Mononuclears from fresh blood. Note radial arrangement of the mitochondria.

FIG. 12. Nucleated red blood-cells with small vacuoles that stain with neutral red. Fresh blood.

FIGS. 13, 14. Lymphocytes in 24-hour cultures with neutral red.

FIGS. 15, 16. Somewhat clasmatocyte-like mononuclears in 24-hour culture.

FIG. 17. Epithelioid-like mononuclear in 24-hour cultures; fat globules prominent.

FIGS. 18, 19. Clasmatocytes in 3-day cultures, neutral red. The large, freshly ingested red cell in figure 19 did not take the neutral-red stain.

FIG. 20. Clasmatocyte in 4-day culture with large ingested red blood-cells, partly digested stuff and fat globules. Nucleus obscured; red blood-cells more or less laked.

FIG. 21. Epithelioid cell from 5-day culture. Neutral red and Sudan III.

FIGS. 22 to 25. Four hypertrophied mononuclears from a 3-day culture with ingested and partly digested red blood-cells, pigment granules and fat globules.

FIG. 26. Epithelioid cell from a 10-day culture.

FIG. 27. Elongated clasmatocyte from a 10-day culture.

FIG. 28. Migrating eosinophile from a 5-day culture.

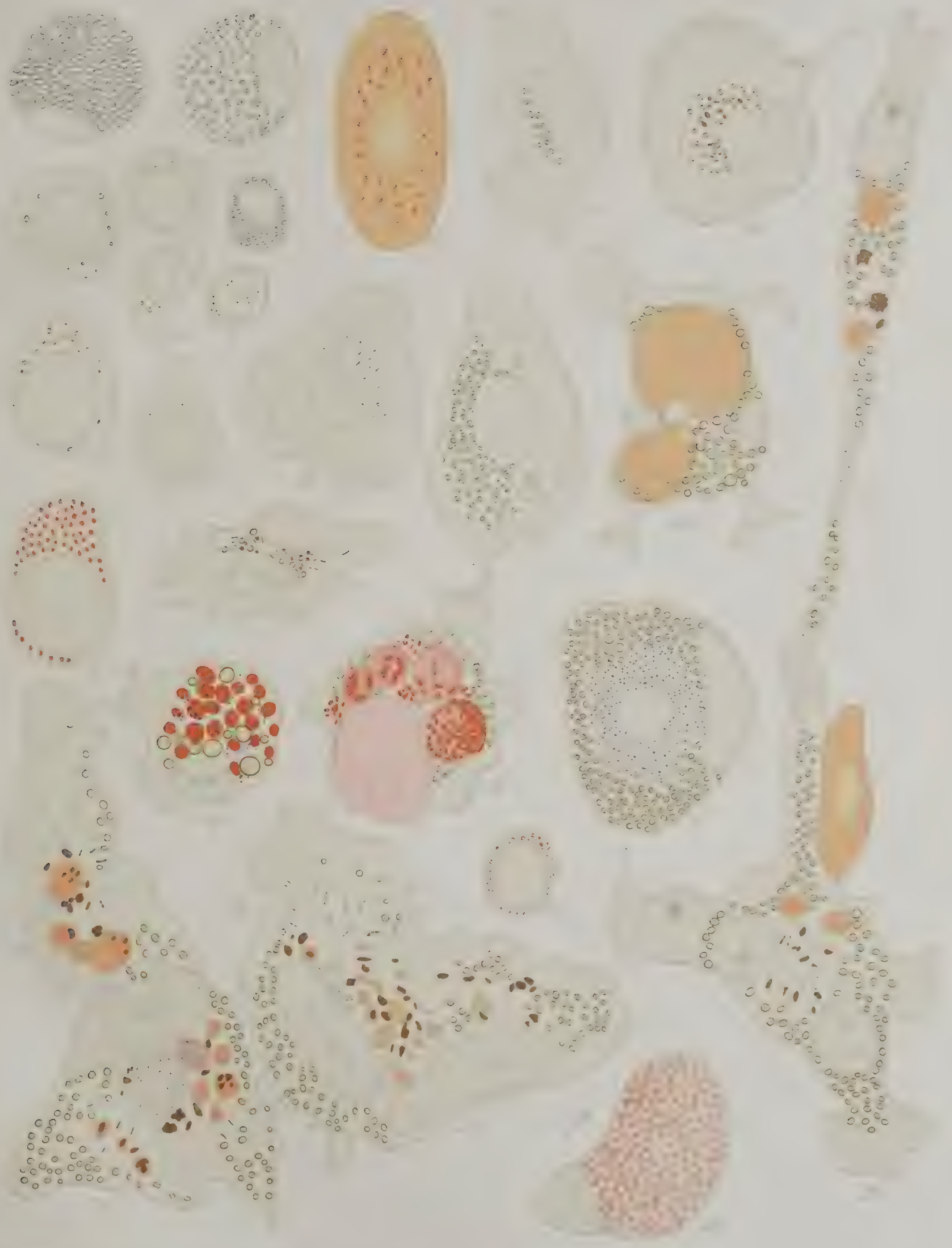

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PLATE 2.

SKATE.

FIGS. 29 to 31. Eosinophilic granulocytes in fresh culture with neutral red. Two of the three vacuoles seen in figure 31 developed while the cell was under observation.

FIGS. 32 to 34. Mononuclears with neutral red in fresh culture. Cells actively migrating.

FIGS. 35, 36. Mononuclears with janus green in fresh culture; migrating.

FIG. 37. Lymphocyte (?).

FIGS. 38, 39. Red blood-cells with neutral red and with janus green.

FIGS. 40, 41. Granulocytes from 10-day culture migrating actively with a few fat globules.

FIGS. 42 to 47. Clasmatocyte type of enlarged mononuclear containing ingested red blood-cells in various stages of digestion; fat globules, and pigment granules; neutral red on cover-glass at time culture was made. 10-day culture. Many of the cells were living 10 days later.

FIGS. 48, 49. Clasmatocytes from 12-day culture, containing many red blood-cells, partly digested debris, and fat globules. The cells are so crowded with ingested stuff that the nuclei can not be seen.

FIG. 50. Red blood-cell in 10-day culture.

FIG. 51. Lymphocyte from a 2-day culture with increased number of granules.

FIGS. 52 to 56. Small macrophages from same culture. These cells were changing shape.

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PLATE 2

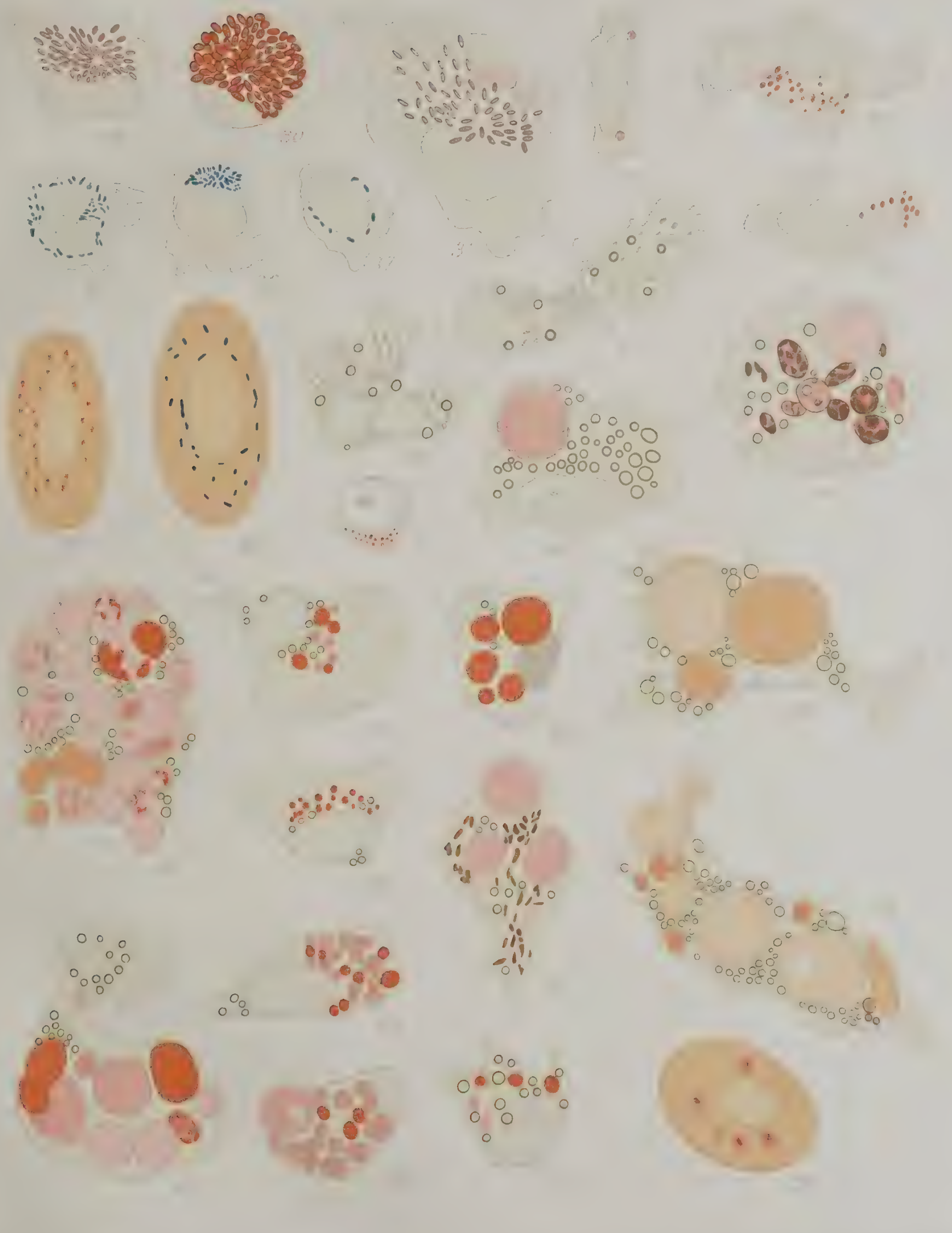

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PLATE 3.

Sculpin.

Fig. 57. Eosinophile with neutral red, fresh culture.

Figs. 58 to 69. White blood-cells, fresh culture with neutral red. The cells are small and difficult to classify. Figures 58 to 63 are probably lymphocytes and 64 to 69 are probably mononuclears. It is difficult to separate these cells into two distinct types.

Fig. 70. Red blood-cell from same preparation.

Fig. 71. Red blood-cells from 48-hour culture.

Figs. 72 to 74. Epithelioid cells, 48-hour culture. Enormous hypertrophy in 48 hours. Figure 73 epithelioid cell with 3 recently ingested red blood-cells and fat globules. Figure 74 the same cell shortly afterwards. Half hour later the cell had again spread out somewhat as in figure 73.

Fig. 75. Binucleated marcrophage from same culture.

Fig. 76. Epithelioid giant cell with 3 nuclei, from the same culture. The neeele-like bodies are probably distorted red blood-cell membranes. Many small fat globules in all the cells.

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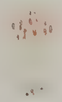

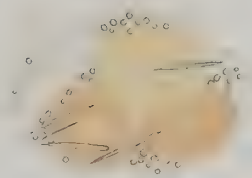

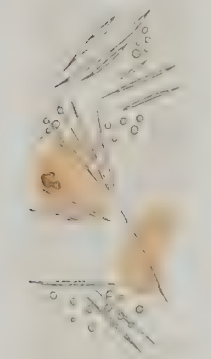

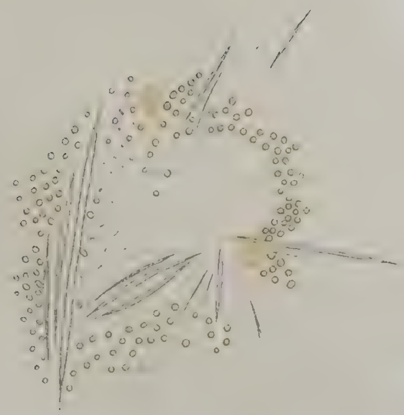

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PLATE 4.

Sculpin (Figures 77 to 81).

Figs. 77, 78. Elongated epithelioid cells from a 9-day culture.
Figs. 79 to 81. Clasmatocytes from an 8-day culture. Neutral red for 2 days.

Cunner (Figures 82 to 89).

Figs. 82, 83. Epithelioid cells from a 12-day culture of cunner's blood. Each cell contains a finely granular centra area, surrounded by a zone of fat globules, and a single ingested red blood-cell.
Fig. 84. Giant cell from a 6-day culture of cunner's blood.
Fig. 85. Lymphocyte, fresh culture.
Fig. 86. Granulocytes, fresh culture.
Fig. 87. Mononuclear, fresh culture.
Fig. 88. Red blood-cell.
Fig. 89. Binucleated epithelioid cell from a 12-day culture.

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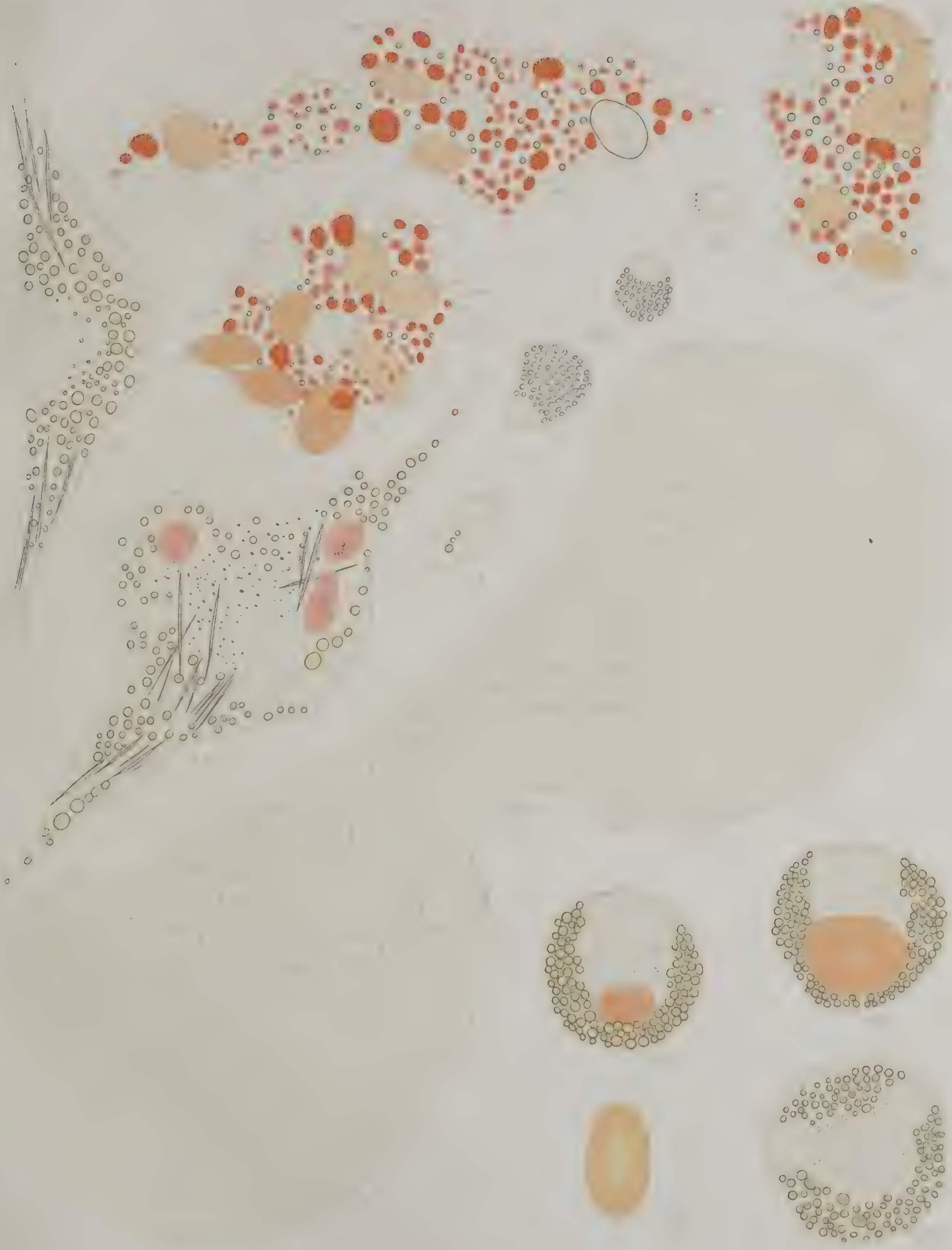

PLATE 5.

Toad.

Fig. 90. Eosinophile from a fresh culture with neutral red.
Figs. 91, 92. Mononuclears from the same culture.
Fig. 93. Red blood-cell from the same culture.
Figs. 94 to 96. Lymphocytes from the same culture.
Figs. 97, 98. Polymorphonuclears from the same culture.
Figs. 99, 100. Epithelioid cells from a 3-day culture with neutral red.
Fig. 101. Intermediate form between epithelioid and clasmatocyte from the same culture.
Fig. 102. Clasmatocyte with ingested red blood-cells, from the same culture.
Fig. 103. Epithelioid cell, from a 5-day culture, with 3 red blood-cells and fat globules.
Fig. 104. Clasmatocyte from the same culture, with four red cells and pigment granules.
Figs. 105, 106. Epithelioid cells from another 5-day culture.
Fig. 107. Epithelioid cell from a 6-day culture, with central area, ingested red blood-cells, and zone of fat globules.
Fig. 108. Thrombocytes or hemoblasts flattened on the cover-glass. Zenker, H. & E.
Fig. 109. Thrombocytes (spindle forms), same culture.
Fig. 110. Thrombocyte (changing to red blood-cell ?), same culture.

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PLATE 5

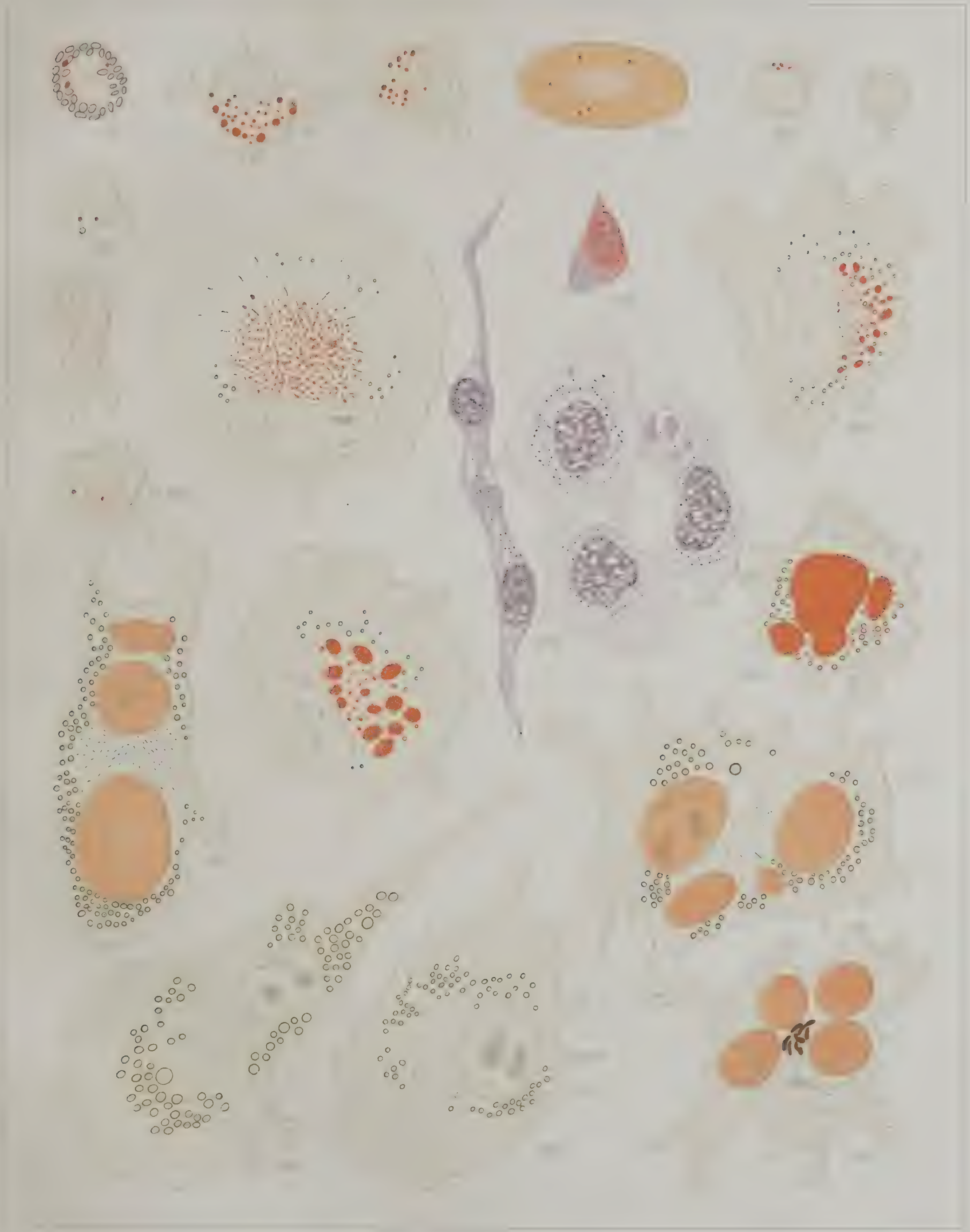

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PLATE 6.

TOAD.

FIG. 111. Epithelioid cell from a 3-day culture.
FIGS. 112, 113. Epithelioid cells with large fat globules; from a 16-day culture.
FIG. 114. Clasmatocyte from the same culture.
FIG. 115. Giant cells with neutral red; 10-day culture. ×750.
FIG. 116. Epithelioid cell same culture. ×750.

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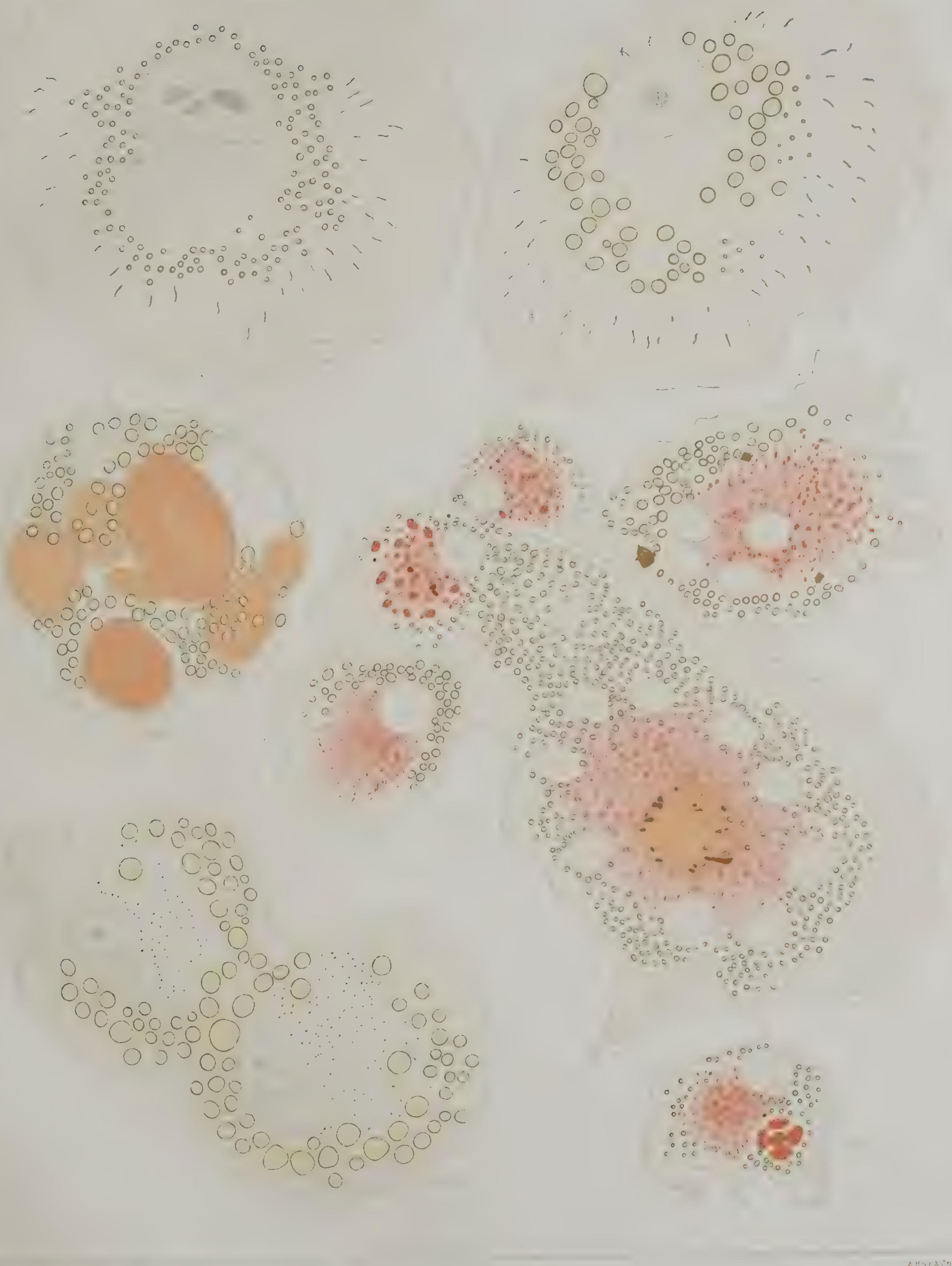

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PLATE 7.

Leopard Frog.

Fig. 117. Lymphocytes.
Fig. 118. Binucleate mononuclear, fresh culture.
Fig. 119. Mononuclear.
Fig. 120. Mononuclear migrating.
Fig. 121. Polymorphonuclear neutrophile.
Fig. 122. Eosinophile, 24-hour culture.
Figs. 123, 124. Mononuclears, same culture.
Fig. 125. Red blood-cell, 24-hour culture.
Fig. 126. Macrophage with many ingested red blood-cells and a few fat globules; from a 4-day culture.
Fig. 127. Epithelioid cell with 3 ingested red blood-cells and many fat globules; from a 7-day culture.
Fig. 128. Epithelioid cell from same culture with mitochondria and fat globules radially arranged about the central body.
Fig. 129. Giant cell from a 7-day culture.

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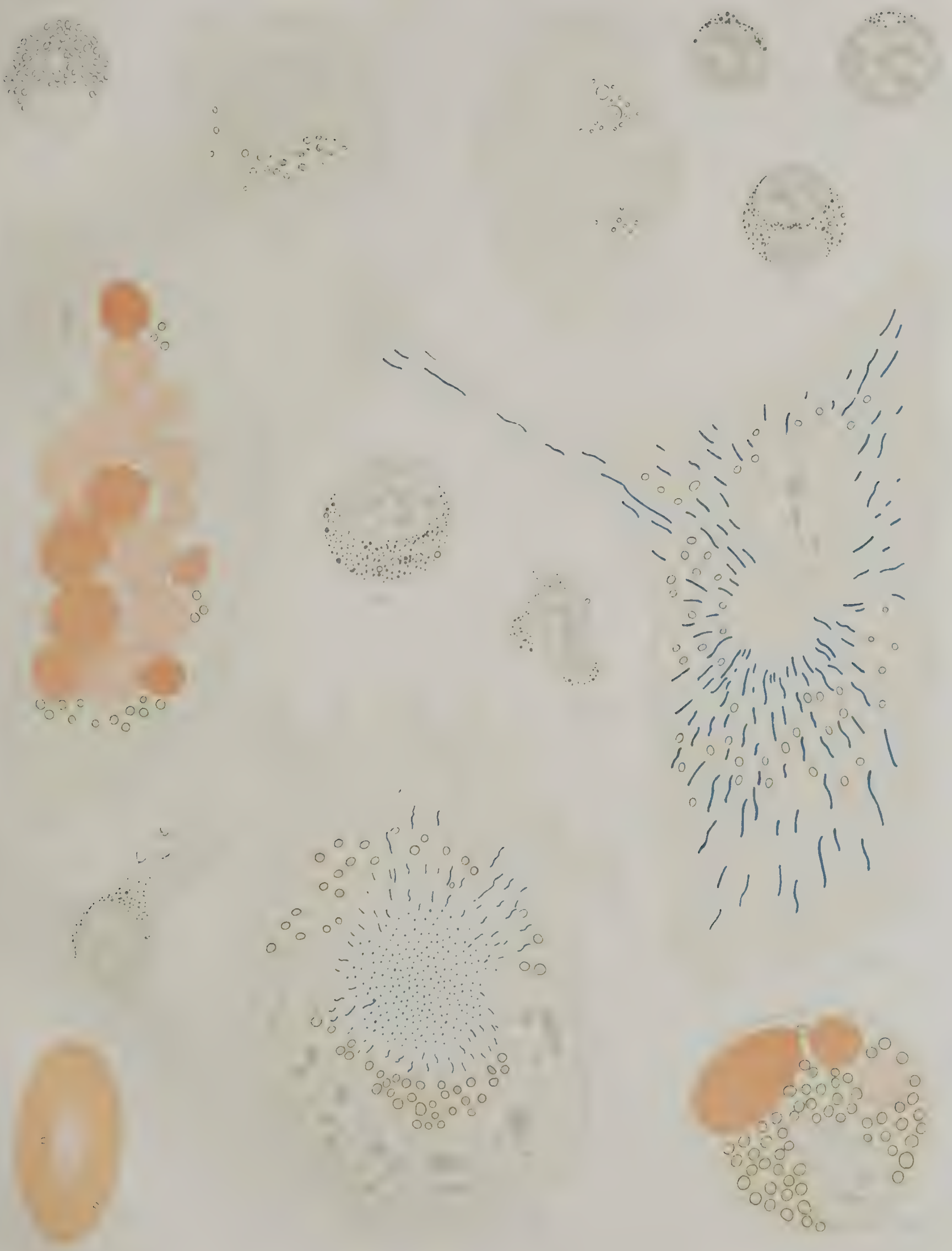

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PLATE 8.

Garter Snake (Figures 130 to 141).

Figs. 130, 131. Eosinophiles in a fresh culture.
Fig. 132. Mononuclear, fresh culture.
Fig. 133. Migrating lymphocyte, fresh culture.
Fig. 134. Red blood-cell, fresh culture.
Figs. 135 to 137. Epithelioid cells, 7-day culture. In figure 135 a freshly ingested red blood-cell lies against the nucleus.
Figs. 138, 139. Epithelioid cells, 13-day culture with neutral red.
Figs. 140, 141. Clasmatocyte-like cells from the same culture.

Green Grass Snake (Figures 124 to 149).

Figs. 142 to 145. Epithelioid cells, from a 3-day culture, with neutral red. Note the freshly ingested red blood-cells against the nucleus in figure 144.
Fig. 146. Clasmatocyte, same culture.
Figs. 147, 148. Eosinophiles, same culture.
Fig. 149. Red blood-cell, same culture.

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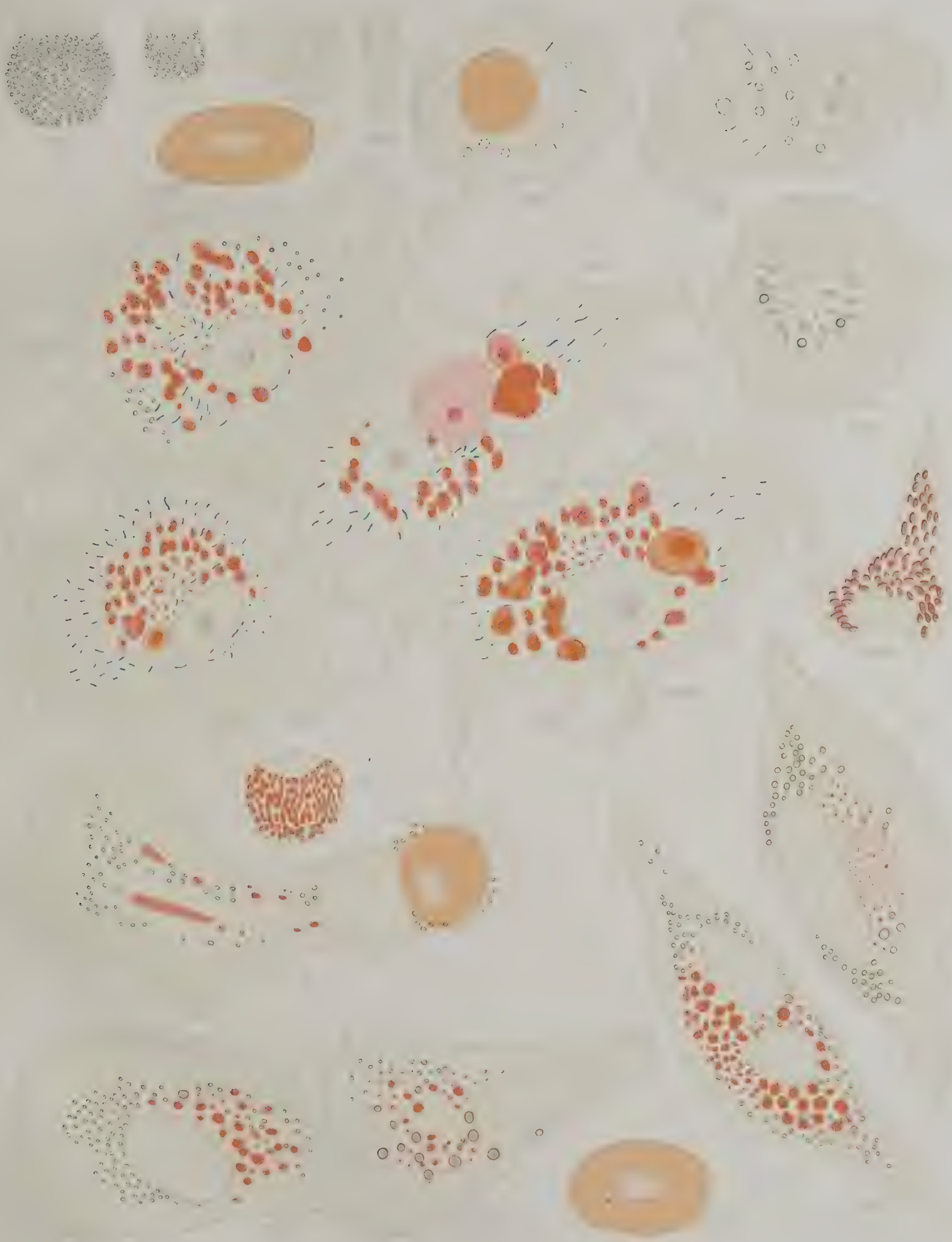

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PLATE 9.

LIZARD.

FIGS. 150, 151. Migrating eosinophiles with spindle-shaped granules, 5-hour culture.
FIGS. 152, 153. Eosinophiles with round granules, 5-hour culture.
FIGS. 154 to 156. Lymphocytes, same culture.
FIGS. 157 to 159. Mononuclears, 5-hour culture.
FIG. 160. Red blood-cell, 5-hour culture.
FIGS. 161 to 163. Epithelioid cells, 6-day culture.
FIGS. 164, 165. Macrophages, same culture
FIG. 166. Eosinophile, same culture.
FIG. 167. Lymphocyte, same culture.

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PLATE 10.

Lizard.

Figs. 168, 169. Macrophages, 8-day culture, with ingested red blood-cells. The nuclei of the macrophages are obscured by the ingested material and fat globules.
Fig. 170. Epithelioid cell, same culture.
Fig. 171. Giant epithelioid cell with 3 nuclei, same culture.
Figs. 172. Elongated epithelioid cell, same culture.
Figs. 173 to 176. Eosinophile, lymphocyte, thrombocyte (?), and red blood-cell, same 8-day culture.

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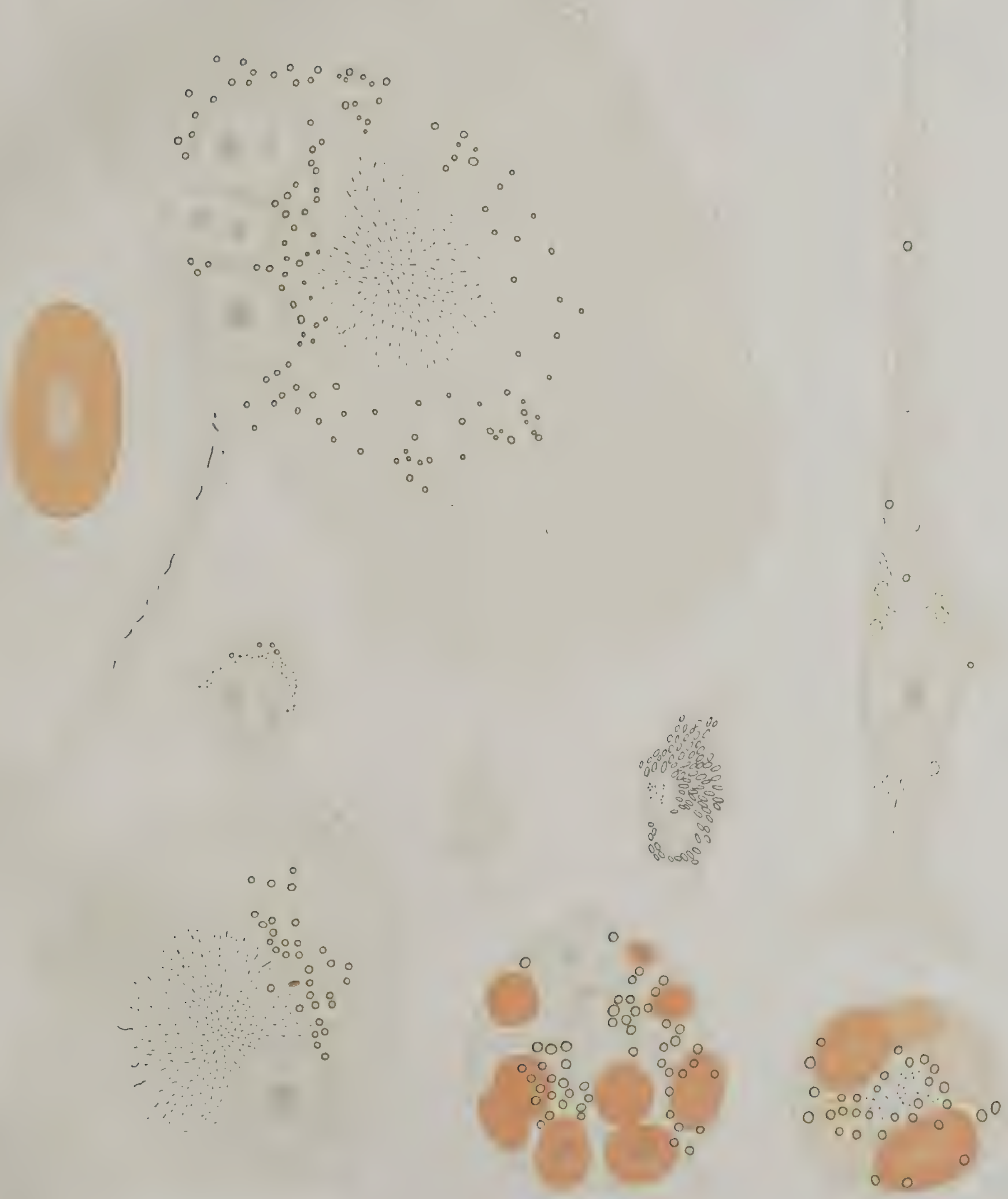

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PLATE 11.

LIZARD.

FIG. 177. Elongated epithelioid cell from 8-day culture.
FIGS. 178 to 180. Epithelioid cells from a 10-day culture, with neutral red.
FIG. 181. Macrophage, same culture.

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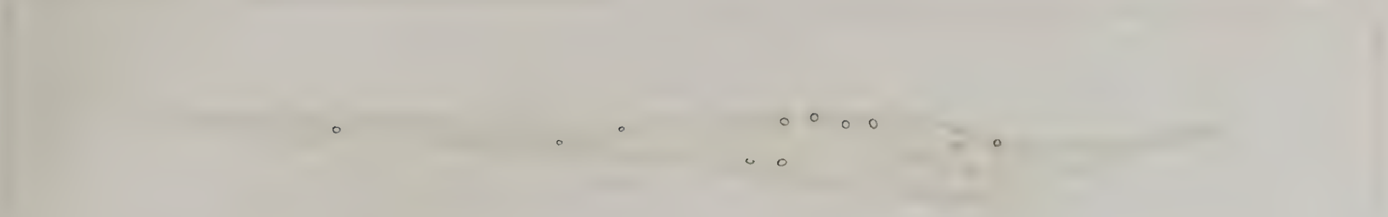
PLATE 11



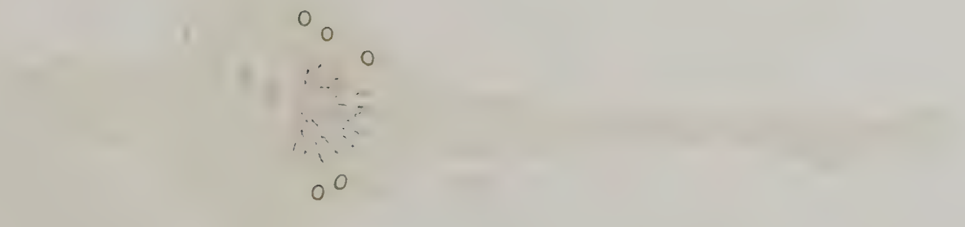


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PLATE 12.

LIZARD.

FIG. 182. 12-day culture of lizard's blood. A patch of macrophages, epithelioid cells, and giant cells on the cover-glass. The macrophages are crowded with ingested red blood-cells and are dark in this photograph.

FIG. 183. A group of giant cells from the same culture. ×480.

FIGS. 184. Epithelioid cells on the cover-glass of a 16-day culture showing variations in form. The superficial layer is spread out into a thin membrane. The granular mitochondria are irregularly distributed in the intermediate zone, which was composed mostly of fine fat globules scarcely recognizable in the photographs. Two clasmatocyte-like forms. Janus green, iodine vapor. ×480.

FIG. 185. Elongated epithelioid cells that are fibroblast-like in general appearance, same culture. ×480.

FIG. 186. Giant cell, same culture. ×700.

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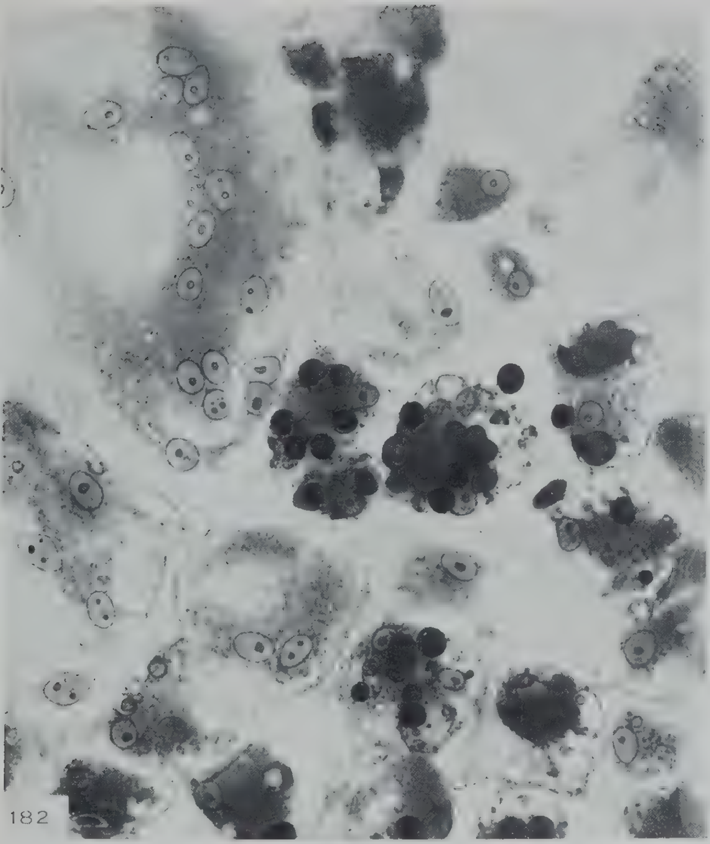

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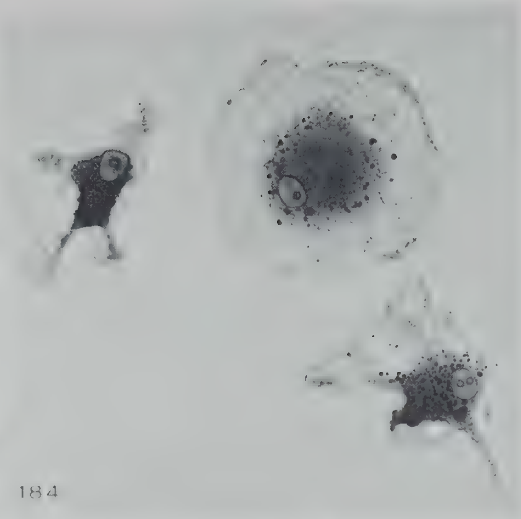

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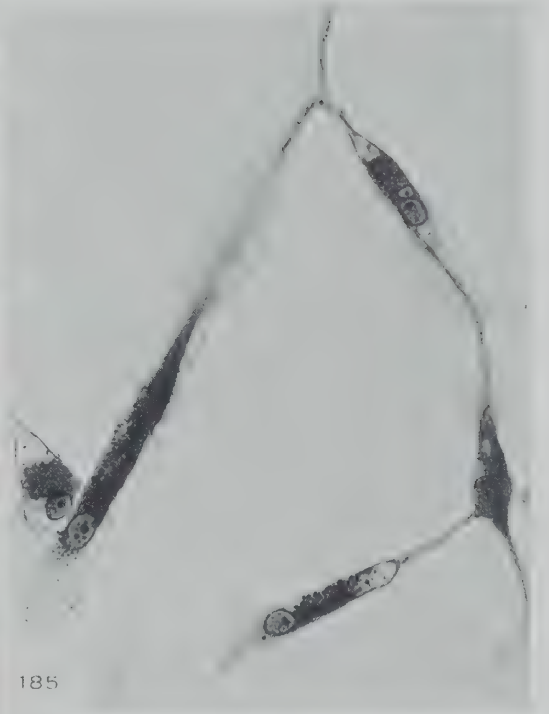

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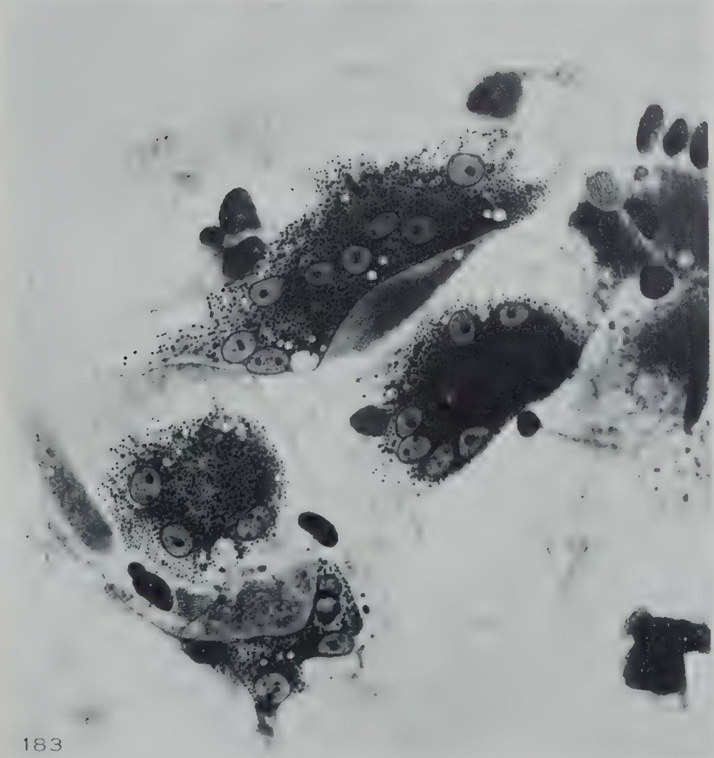

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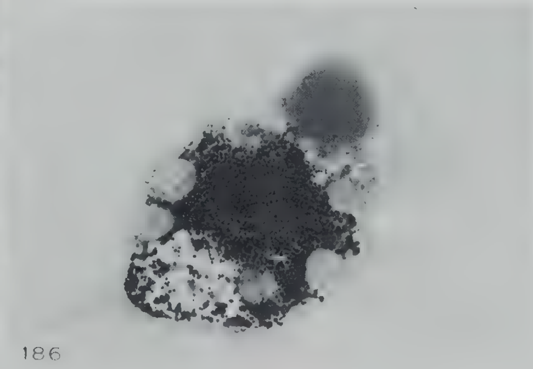

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PLATE 13.

LIZARD.

FIG. 187. Epithelioid cells showing variations in form. Granular mitochondria mostly in the intermediate zone From same culture as Figure 184. × 480.

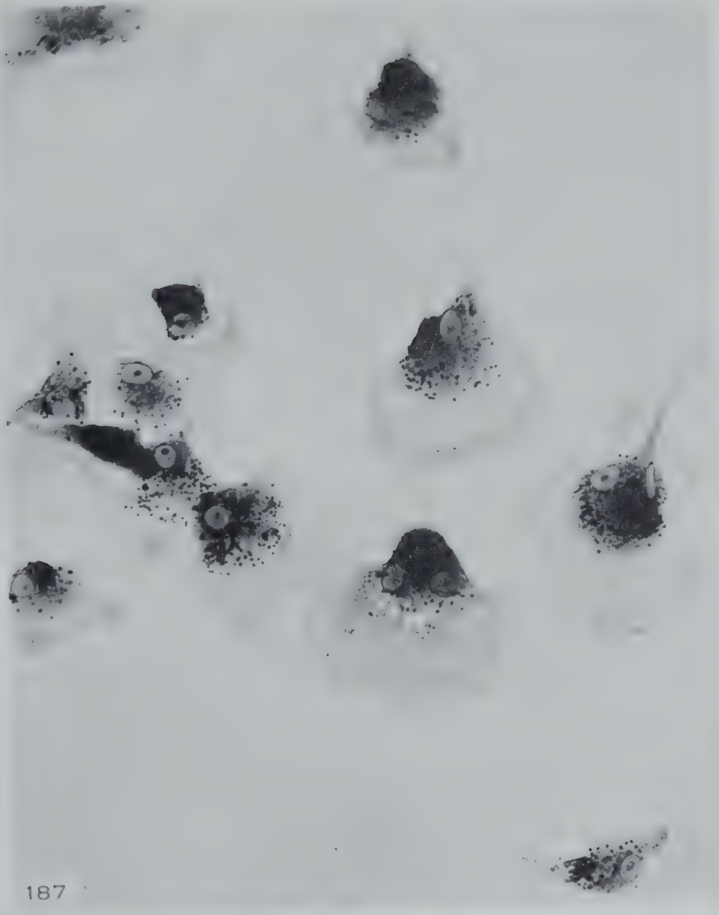
FIG. 188. Elongated epithelioid cell and small giant cell showing film-like pseudopodia. Iodine vapor. ×700.

FIG. 189. Migrating cell from a 17-day culture showing rod-shaped mitochondria. Janus green, iodine. ×1666.

FIG. 190. Two epithelioid cells heavily laden with neutral red staining granules from a 34-day culture. Neutral red, iodine. ×700.

FIG. 191. Thrombocytes from a 16-day culture. The cell in the center shows indications of transformation into a red blood-cell. Mitochondrial granules are clearly shown. Janus green, iodine. ×1450.

FIG. 192. Elongated epithelioid cells from same culture shown in figure 187. These forms might easily be mistaken for fibroblasts. × 480.



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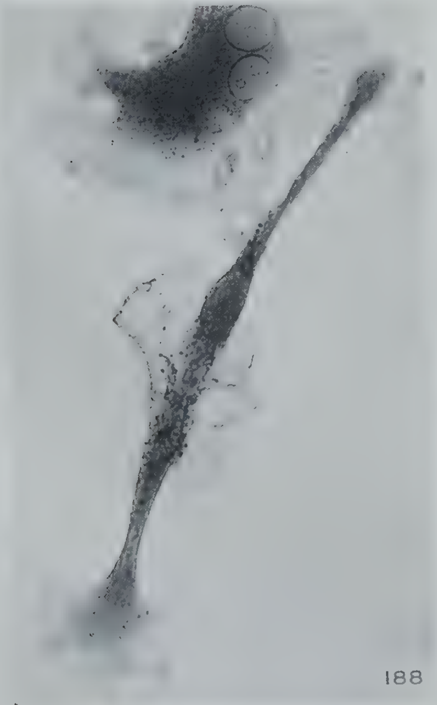

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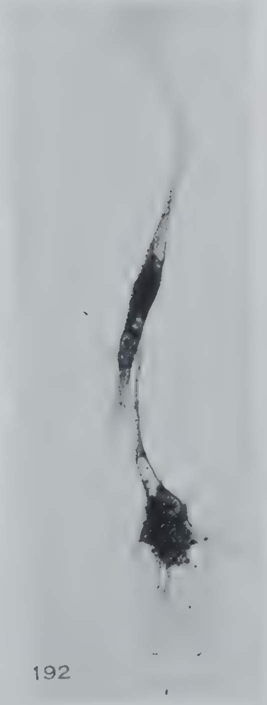

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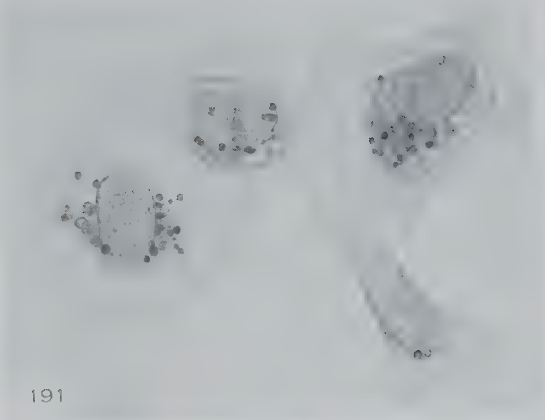

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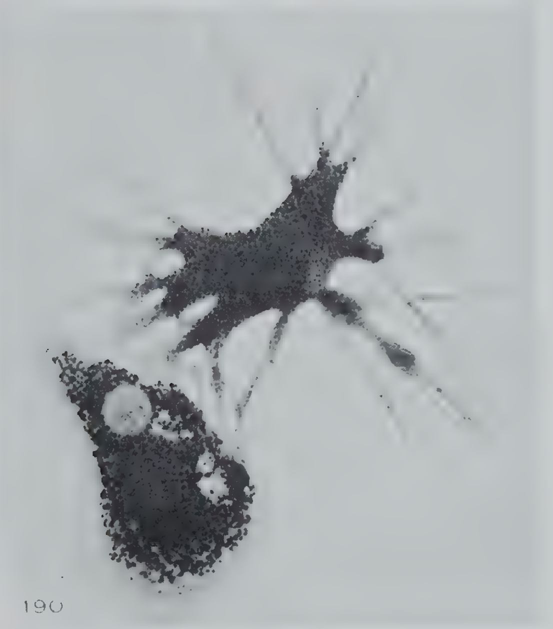

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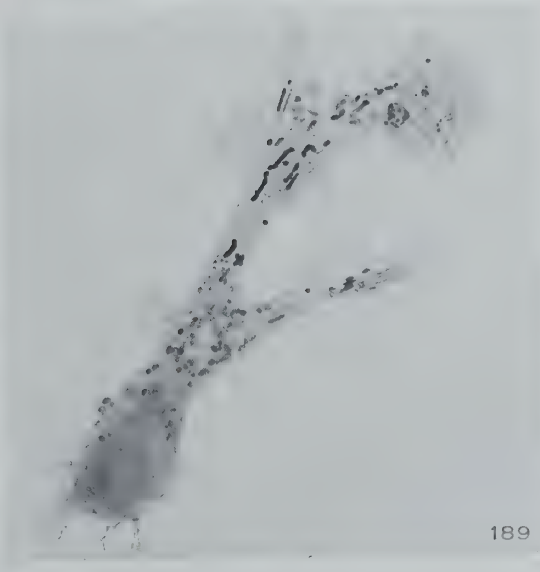

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CONTRIBUTIONS TO EMBRYOLGY, NO. 97.

ORIGIN OF THROMBOCYTES AND OF THE DIFFERENT TYPES OF BLOOD-CELLS AS SEEN IN THE LIVING CHICK BLASTODERM.

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With one plate and one text-figure.

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ORIGIN OF THROMBOCYTES AND OF THE DIFFERENT TYPES OF BLOOD-CELLS AS SEEN IN THE LIVING CHICK BLASTODERM.

INTRODUCTION.

The mode of hematogenesis has been a most puzzling question, as evidenced by the numerous views and theories that have grown up—indeed are still growing up—around it. In the first place, there are the two major concepts: one, the origin of all blood-cells from one common stem, the other, the separation of red cells from white cells by a distinct ancestor for each. In the second place, there are a number of theories concerning the origin of the blood-cells that are by no means settled; among these, three are at the present time of especial interest.

(1) There are still confused and conflicting views on the question of the differentiation of the platelets and thrombocytes. While it is now rather generally accepted that the platelets in the blood of mammals are produced by the constriction of the cytoplasm of megakaryocytes, there have been put forth six entirely different concepts regarding the differentiation or nature of the nucleated thrombocytes in the blood of birds and cold-blooded vertebrates. One group believes that thrombocytes are descendants of lymphocytes or primitive lymphoid cells, while others believe in their endothelial origin. A third group has postulated that thrombocytes are damaged or degenerated red cells; others again regard them as a transitory form of lymphocytes. Furthermore, a number of authors have endowed these cells with hemoblastic nature, considering them capable of developing into red cells, while a few workers regard them as a type of giant cell.

(2) The question as to the method of development of the clasmatocytes or histiocytes in the blood and the tissues does not seem to have been completely settled. Although nearly all investigators have agreed that the primitive endothelial cells are able to give rise to clasmatocytes, views differ as to whether or not mesenchymal cells also possess such properties.

(3) The mode of development of monocytes has hitherto been one of the enigmatic problems. The discovery by Sabin, Cunningham, and Doan (1925) that the monocyte, as seen in living form, is characterized by a marked permanence of the rosette of vitally stainable particles around the centrosphere, has thrown new light upon this question. These authors believe that the monocyte is a distinct strain from the endothelial phagocyte or clasmatocyte and arises outside the blood-vessels from reticular cells.

It has not been my purpose in the present study to deal with all these questions, but I have attempted to find out how much the course of the formation of the thrombocytes and of the white blood-cells could be seen in supravitally stained chick embryos during the first 7 days of incubation.

METHODS.

In this study I have used three kinds of preparations: First, the entire living blastoderm of the chick, stained with neutral red, janus green, or a combination of the two, and mounted in a hanging-drop preparation; second, drops of blood with supravital staining; third, fixed films of blood with Wright's stain.

The method of studying the entire blastoderm in hanging-drop preparations was first used by Miller and McWhorter (1914), who grew the specimens in clotted chick plasma. I have followed the simple technique described by Sabin (1920), which consists in mounting the blastoderm in a solution developed by W. H. and M. R. Lewis and called Locke-Lewis solution. The entire chick can be used during the first three days of incubation; after that, only the yolk-sac. Following the directions of Sabin, I have used the Locke-Lewis solution somewhat modified, according to the age of the chick, using 1.04 per cent NaCl for chicks of the second day of incubation, 1.0 per cent for the third day, and the usual 0.9 per cent for chicks of the fourth day and older. For distinguishing slight traces of hemoglobin, there is some advantage in using the simple Locke solution, since it lacks the slight yellowish tinge of the bouillon.

For the supravital staining of the hanging-drop specimens a 1.0 per cent aqueous solution of neutral red or of janus green (1 to 3 drops for a weak solution, 5 to 10 drops for a strong solution, or 10 to 12 drops for a very strong solution) is added to 10 c.c. of Locke or Locke-Lewis solution, in the second dish of fluid in which the chick is placed before mounting. The chick is left in the solution of stain, 10 to 30 minutes for the neutral red and from 1 to 2 hours or more for the janus green, before the specimen is mounted in the hanging drop. The time needed for staining differs greatly according to the type of cell to be studied.

For making films of blood, I followed the usual technique of drawing the blood in hair-like capillary glass tubes. The actual piercing of the vessels and drawing blood from the lumen is easy from the end of the second day of incubation on, but it is more difficult to secure the blood during the second day of incubation. The following method proved to be the most successful even at the stage of 14 somites. After having mounted the blastoderm on a cover-slip with the ectoderm against the glass, under the microscope the point of a fine glass capillary is put into the space between the ectoderm and entoderm, and one or two relatively large vessels are broken. The blood running out of the vessel should be drawn quickly into the glass capillary. Sometimes this method is so successful that almost pure blood is obtained, and, fortunately too, there exist at this stage practically no free cells in the meshes of the vascular plexus, with the exception of a few endodermal wandering cells (only in the anterior part of the area pellucida) and a lesser number of clasmatocytes, which would never confuse the results of this experiment.

At the time the differentiation of blood-cells is going on only in the area opaca, that is to say, when the chick has about 8 to 11 somites, I injured the blood-vessels in this area with a glass capillary and drew the fluid, including not only the blood-cells but also endodermal cells and many yolk-globules. Such mixing, however, does not always hinder the observation of cells in the slide smears, especially for the hemoglobin test. I did, in two cases, produce relatively good cover-slip specimens of such blood stained with Wright's solution.

The blood obtained by these methods was examined in three ways: in fresh films without any stain but ringed with vaseline; in films with single or double supravital stains; films on dried cover-slips with Wright's eosin-methylene blue. For preparing the slides with the supravital dyes I used Sabin's (1923) technique, but used half the strength of neutral red adapted to adult cells, since the full strength stained the nuclei and also caused too ready a precipitation of the basophilic substance of the primitive red cells.

SUPRAVITAL STAINING OF MESODERM, ANGIOBLASTS, BLOOD-ISLANDS AND BLOOD-VESSELS.

In these studies with chick blastoderms I have been able to confirm the observations of Sabin (1920) concerning the differentiation of angioblasts from mesenchyme, the formation of the plexus of solid angioblasts and their transformation into vessels by a liquefaction of the central mass of cells, leaving the peripheral angioblasts to become endothelium. There are three zones of mesoderm in the area pellucida against which the angioblasts can be studied: First, the area of large vesicles which, by fusion into a single cavity, give rise to the amnio-cardiac cavities; second, the fine network of mesoderm of the middle and posterior zones of the area pellucida; and third, the axial zone of the somites and the dense, undifferentiated mesoderm posterior to them.

In these zones the primitive mesodermal cells are usually polygonal in shape, furnished with long or short processes which are connected with those of neighboring cells, thus forming syncytial plexuses, dense or sparse, corresponding to the density of the cells. Besides the mesoderm, which is underneath the plexus of angioblasts, as seen in the living specimen mounted with the endoderm against the cover-slip, there are a few mesenchymal cells in the meshes of the angioblastic network. These cells have a clear, homogeneous cytoplasm containing usually a few refractive granules, or sometimes one or two granules of yolk. If the cells are in perfect condition, their nuclei are not recognizable, but when the cells are dying, as is commonly the case with an embryo exposed to the stains, the oval or round contours of their nuclei, as well as one or two enclosed nucleoli, become readily visible in the cytoplasm. The mesodermal cells and the more differentiated mesenchymal cells are much more distinctly seen in the region of the head and, after the fourth day of incubation, also in the tissues of the allantois, amnion, and chorion.

The primitive mesodermal and mesenchymal cells react in nearly similar manner to vital dyes. With janus green they exhibit many tiny, round, stained granules, or sometimes short rods of mitochondria scattered throughout the cytoplasm, around the nucleus, or even in the long processes (fig. 1). On the other hand, these cells seldom show any reaction to neutral red; an occasional cell may have one or two granules that stain with neutral red, but they are never as constant a finding as those which stain with janus green. When the embryo was left in a strong solution of neutral red for more than 2 hours there appeared several stained granules in many of the mesoblastic cells of the area pellucida, and especially in the allantois and chorion, but this phenomenon is not to be taken for a true vital staining.

When such mesoblastic cells differentiate into angioblasts, however, there follow striking changes in the cytoplasm, not only in its appearance but also in its reactions to vital dyes. In the living specimen the isolated cells or the dense cord of angioblasts are readily distinguishable from the undifferentiated mesoderm beneath, by such characteristics as the refractivity or greater density caused by an increase of granules in the cytoplasm. In the cords of angioblasts the nuclei may not be seen in the living state, though they may be inferred by the circular arrangement of the refractive granules.

The difference between the mesoblastic and angioblastic cells is much more manifest in their affinity for vital dyes. The angioblasts take up neutral red easily and promptly from its weak solution. The stained granules are generally small, but sometimes reach the enormous size that is usually to be seen in stained endodermal cells. Such red granules, mingled among the unstained refractive granules, are arranged in the narrow zone of protoplasm around the nucleus in such a way as to indicate the position of an invisible nucleus. Figure 2 shows a newly formed blood-island reacting in the same manner to neutral red. In some cases, in which the contours of the individual angioblasts in the cord were somewhat clear, a large number of neutral-red granules were seen grouped on one side of the nucleus, though not in the form of a distinct rosette. As a consequence of their being so sensitive to neutral red, the network of angioblasts is readily distinguishable by its reddish color, even under a low magnification of the microscope.

With janus green the angioblasts show tiny mitochondria scattered through the cytoplasm, intermingled with the refractive granules or droplets. It takes a much longer time, however, to obtain the mitochondrial staining; moreover, the color exhibited a tendency to fade out rather soon, showing a weaker affinity for janus green. This might be due partly to the difficulty which this stain has to encounter in penetrating the angioblasts through ectoderm and endoderm.

The formation of the lumen of the blood-vessel by the liquefaction of angioblasts, the formation of red blood-cells from both angioblasts and endothelium, described by Sabin (1920), and the identification of the megaloblast as the first or first few generations from endothelium toward the formation of red cells (Doan, Cunningham, and Sabin, 1925) I have amply confirmed. During the time of the liquefaction of angioblasts and in the early blood-vessels there is to be noted a special type of cell which can never be considered either as a megaloblast or as any other kind of primitive blood-cell. They are exceptionally small, the smallest cells found in the blood-vessels until the second day of incubation. They have spherical form and generally neither possess pseudopodia nor show amœboid activity. Their small, semicircular or kidney-shaped nuclei, without nucleoli, are always located eccentrically in the cytoplasm. The greater part of the cytoplasm is occupied by a number of refractive granules of nearly equal size, but sometimes one sees one or two vacuoles which are so perfectly clear that the cell looks as if there were a hole in the cytoplasm, when the vacuole is in the center of the cell, or as if the cell had a defect, when the vacuole appears along the cell membrane. In this latter case the vacuole is usually larger than the one found in the central part of the cell, and only by careful watching can one discern the thin layer of cell membrane along the outside of the vacuolar space corresponding to the supposed defect. The appearance of the vacuoles is considered as precursive of the fate of these cells, namely, their breaking up into fragments. At the stage in which the primitive vessels have already been formed but the circulation not yet begun, such debris of broken-up cells is often found scattered in the lumina of the vessels while they are undergoing cytolysis or are picked up by the phagocytes. Not only cell debris but also whole cells are frequently seen to be engulfed by the phagocytes.

Cells of this type stand out in sharp contrast to any other primitive blood-cells and to the megaloblasts in their reaction to neutral red. Even in a strong solution of this stain they present no red granules, while many such appear in the primitive red cells. Only in exceptional cases did I find in them a few neutral-red bodies, and this finding is quite reasonable if we take into consideration that these cells are derived from the angioblasts, which show, as was above stated, many neutral-red bodies. An analogous phenomenon is observed between the so-called endodermal wandering cells and the endodermal cells, the latter of which have been derived from the former. These cells, destined to degenerate, are of constant occurrence in the middle part of the area pellucida, though quite variable in number; in some cases they were found in such abundance as almost to fill up the lumen of a vessel. In other specimens only one or two were found floating in a vessel, often in company with primitive blood-cells. These small, round cells with refractive granules are found not only within the lumina of vessels, but even in the solid bands of angioblasts, at the stage of 9 to 12 somites. They become free as vacuolation is carried out in the cells surrounding them, and consequently represent the first cells occurring in the lumen of the vessel. In the anterior and posterior parts of the area pellucida they are found only in small numbers and always accompanied by the primitive blood-cells or blood-islands.

It seems quite certain that most of these degenerating cells are derived directly from angioblasts. I should say, however, that in the living specimens, especially in the later stage, they showed a slightly yellowish color, which might be regarded as indicating some relationship between these cells and the megaloblasts. If the angioblasts had already acquired any hemoglobin, as believed by Van der Stricht, such coloring of the cells in question would be a matter of course, but I could not obtain sufficient evidence of this, at least at the stage of the angioblastic network, although some of the blood-islands in the latter period have evidently accumulated a small amount of hemoglobin before the separation of the individual cells. I should rather, therefore, take these degenerating cells with hemoglobin for an abortive type of primitive red cells, for it is not difficult to find those cells that are considered as transitional between the two. These small cells were described by Sabin (1920, p. 254) as dead cells, and they seem also to coincide with what has been illustrated by Dantschakoff (1908, Taf. 28, fig. 5) as degenerating lymphocytes.

Besides such small degenerating cells there is occasionally found a large round cell with distinct outline and dense protoplasm, imbedded in the cord of angioblasts of the anterior and middle parts of the area pellucida. With the liquefaction of the surrounding mass of cells, this round cell is set free in the lumen of the vessel and then begins to divide, to differentiate further into the primitive red cell. Such large cells are found only in the very beginning of blood-vessel formation and show apparently no tendency toward developing into blood-islands, although they have precisely the same character as the primitive blood-cells that are about to separate from the chain of endothelial cells into the lumen, except that the latter often develop into blood-islands by the proliferation of the nucleus. In fact, this primitive blood-cell, derived directly from the angioblast, has only a minor part in the formation of blood-cells.

Returning to the problem of the formation of blood-vessels, it is of considerable interest to observe the process of vascularization in the posterior part of the area pellucida, as contrasted with that in the anterior and middle parts. Here the earliest vacuolations take place, usually near the outer layer of angioblasts. While the cells of this layer flatten out and become converted into an endothelial wall, the vascular spaces spread out to each side in a split form, then fuse with the neighboring one to form a continuous lumen, at the same time leaving in it a large cell-island connected at some point with the endothelial wall. This cell-island is the so-called blood-island in a true sense. In the adjacent vessels, of course, the whole central mass undergoes liquefaction, constituting the common type of blood-vessels. As stated above, blood-islands, in the early stages, react in a nearly similar manner to vital dyes, and with neutral red show many large and small granules in the cytoplasm (fig. 2). At a later stage, when the isolation of blood-cells begins, one always meets with obstacles in attempting to secure supravital staining of the blood-islands. It can usually be done successfully, however, by using a strong solution of the dye.

If we now compare the mechanism of vascularization in these three parts of the area pellucida, there can be observed a distinct division of work. In the posterior part, where the massive angioblasts are formed, not all the cells are sacrificed for vascularization; a considerable number of them remain intact and undergo further differentiation into blood-cells. This is very properly planned for economical metabolism in embryonic life.

In the intermediate zone of the area pellucida, where the primary vessels develop into such large blood-vessels as the omphalo-mesenteric and vitelline veins, the connecting canals between the heart and the area opaca in which the primitive red cells have already been produced in great number, the quick establishment of a vascular system is much more necessary than the formation of blood-cells, in order to remedy the condition described by Kollmann as "blood without embryo and embryo without blood." For this purpose there is, on the one hand, the breaking up of angioblasts into small degenerating cells, in addition to the common process of vacuolation, and on the other the persistence of a few small blood-islands and blood-cells.

The anterior zone of the area pellucida, where the head fold and lateral fold are rising and arching over the embryo and part of which is occupied by the so-called pro-amnion, free from mesoderm, is more or less narrow and seems to have comparatively minor significance in reference to the establishment of the circulatory system and the formation of blood-cells. We observe here the usual process of vacuolation and later a relatively small number of blood-islands and blood-cells floating in the plasma, which is not yet in circulation.

DEVELOPMENT OF RED CELLS.

In the study of hematogenesis, the so-called monism and dualism touch the fundamental point. While such authors as Saxer, Maximow, Dantschakoff, Bryce, Pappenheim and Weidenreich are advocates of the first concept, Bizzozero, Mosso, Mondino, Ziegler, Dehler, Van der Stricht, Engel, Weber, Schridde, Naegeli, Sabin, Cunningham and Doan are supporters of the second. It is far from my purpose to discuss here this extraordinarily extensive literature, but I shall simply cite a few of their views or observations in the course of the description of my own experiment.

Before entering into details, I would like to give some criteria upon which I base my decision as to whether or not a given cell contains hemoglobin.

(First.) We should not at once assume the presence of hemoglobin simply because the cytoplasm has taken the eosin stain, for this stain is not the absolutely specific reagent of hemaglobin. It is the common stain for the cytoplasm, employed in combination with hematoxylin and is used even for staining the nuclei. On the other hand, one should not conclude, without any other test, that a cell has no trace of hemoglobin merely because its cytoplasm shows no red tinge by eosin staining. We know that particles of eosin in its aqueous solution are of amicron size and of such a high grade of diffusibility that we may expect a certain degree of decoloration by post-treatment of the specimen. Moreover, the hemoglobin in a cell can be more or less removed or decomposed by such fixative fluids as alcohol, acid, and formalin.

(Second.) In the observation of living specimens one should not assume the presence of hemoglobin in a cell from its yellowish color. From the time the cell has acquired a definite amount of hemoglobin it is quite easy to recognize the distinct coloring of the cytoplasm obviously due to its presence. Before this period, however, it is extremely difficult to tell whether any cell found in the primitive blood-vessels contains hemoglobin. As is well known, the yolk substance has a slightly yellowish color in the living specimen, and this substance is distributed in visible and invisible form throughout the whole embryonic body, extracellularly and intracellularly, including, of course, the angioblastic cells. Furthermore, the cells have to be examined through the endoderm, in which a large amount of yolk substance is still retained. For these reasons, probably, the typical flattened endothelium in my preparations often showed a yellowish tinge.

(Third.) Even if a certain cellular substance has a yellowish appearance in the living state and stains a reddish color by eosin in the fixed specimen, it need not always be considered as having contained hemoglobin. This is well illustrated in the chick embryo, in which the yolk granules in the endoderm present in the living state a more or less yellowish color, and when fixed can often be stained an intense red by eosin, as has already been shown by Dantschakoff (1908, Taf. 27, figs. 1 and 2). The idea that the staining of yolk globules by eosin is due to Bunge's hæmatogen in them does not seem to be reasonable. At any rate, the staining of hemoglobin by eosin is by no means a true chemical reaction. Not only dyes of small molecules, such as eosin or indigo carmin, but also the acidic dyes of large molecules, such as trypan blue, can, under certain circumstances, stain adult red cells. All these stains, xanthon, indigo, and diazo dye-stuffs, have entirely different chemical constitution.

Hence it is beyond the scope of this work to determine exactly the presence of hemoglobin in each cell found in the primitive blood-vessels. Leaving some points undecided, therefore, I shall proceed with the description of my own observations, which were made under the above precautions and by the following methods: Careful watching of the hanging-drop specimens through the sunlight, often transmitted by blue glass; of the slides of plain and supravitally stained blood drawn from the vascular area; and of the cover-slip smears treated with Wright's stain.

DIFFERENTIATION OF MEGALOBLASTS.

Most of the primitive red cells are derived from blood-islands, but partly, also, from the isolated primitive cells derived directly from angioblasts or indirectly from the endothelial cells. They appear first in the vessels in the posterior part of the area opaca, at the stage when the chick has about 7 pairs of somites, later spreading forward in the vessels along the whole extent of the area opaca. The distinct reddish coloring of this area can be recognized when the embryo shows 9 or 10 somites, not only with lower magnifications but also with the naked eye. This phenomenon has also been illustrated by Kollmann (1884, Taf. XVI, fig. 1) and by Duval (fig. 5, plate 1, in Atlas d'Embryologie). By this time the isolation of megaloblasts has occurred in the vessels of the area pellucida following the progress of vascularization.

In hanging-drop preparations megaloblasts are approximately round, but in reality they present irregular forms, with short, blunt pseudopodia, to which they owe their amœboid movements. While under observation the cells exhibit slow but continuous changes in shape, due to the alternate elongation and retraction of their pseudopodia. Nevertheless, the actual amœboid power of these cells is rather slight as compared with that of the endodermal wandering cells and the clasmatocytes. For example, it took about an hour for a megaloblast to crawl a distance equal to twice the diameter of an adjacent primitive cell. Of course, I could not in this case exclude the cooperation of gravity. Furthermore, it has been shown by Sabin that the changing of cell contours is greatly influenced by the concentration of the fluids.

The large oval or round nuclei, as also the enclosed one or two nucleoli, can be discerned in some cases, while in others they are entirely obscured by granules and dense cytoplasm. When the cells are damaged by the poisonous effect of stains, the outlines of their nuclei and nucleoli become perfectly clear. It is noteworthy that, in spite of the fact that the nucleoli in the living healthy cells are always in the form of round bodies, they change, when damaged, into polygonal form with sharp edges, having stained slightly with the dye. In the cover-slip smears treated with Wright's stain the nucleus appears as a vesicle in the center of the dark-blue cytoplasm. Owing to the scanty chromatin substance, its network is extremely slender and obscure, so that it can be recognized only with the highest magnification. The round or irregular nucleoli likewise stain faintly (figs. 4, 8).

The most important and discussed feature of these cells is the nature of their cytoplasm, and quite numerous views have been advanced as to whether or not this first blood-cell in all hematogenic history already contains hemoglobin. In the living specimens the cytoplasm is dense and contains a few small refractive granules, or, more frequently, medium-sized globules, which appear in the cover-slip smears stained with Wright's solution as perfectly clear spots lacking substance. That such cytoplasm contains a small quantity of hemoglobin has been plainly observed in the living preparations in blood-islands, where the individual cells show a yellowish tint even before their separation. Moreover, I succeeded in drawing the blood from the area pellucida of embryos with 14 somites. In the study of an unstained film of such blood, most of the cells were found to have a yellowish tint. In the cover-slip films stained with eosin-methylene blue, the cytoplasm is dyed intensely bluish with, at the same time, a reddish tinge. In two of the cover-slip preparations of shed blood taken from embryos with 9 somites (fig. 4), the reddish coloring of the cytoplasm was plainly illustrated. These findings are sufficient to justify the conclusion that the primitive red cells contain hemoglobin from the beginning. In the cytoplasm and in the immediate neighborhood of the nucleus, one usually sees a clear red spot. This spot may correspond to the centrosphere, though it is not yet so circumscribed as in the following stage.

If the dilute solution of neutral red be applied to hanging-drop specimens, ordinarily no red granules appear while the cells are in good condition. If, however, the solution is considerably stronger and the cells have taken a step toward death, many granules appear in the cytoplasm, part of them grouped around the nucleus, others about a spot corresponding to the centrosphere, though not in the form of typical rosettes (fig. 3). Such staining occurs more quickly in those cells that have escaped from the blood-vessels by hemorrhage or in the cells of slide smears. Hence the supravital staining of megaloblasts seems to be associated with the regressive metabolism of cells. In other words, the living cells of certain types have the power of resisting the penetration of poisonous dyes. I once demonstrated such a phenomenon in the study of vital staining of amœba proteus. When stained with janus green, the megaloblasts show mitochondria in much the same manner that they show the granules with neutral red, except that the mitochondria are all scattered around the nucleus and are sometimes in the form of short rods. Occasionally, in slides stained with both dyes, a single cell exhibits at the same time a few red granules and many mitochondria (fig. 3).

In slide smears stained with one or both dyes one can see a peculiar phenomenon, which can not, however, be interpreted as any vital process. A tremendous number of granules stained with neutral red or janus green appear and almost fill the cytoplasm, leaving only a narrow, clear, yellowish rim at the periphery of the cell. In this case the usual neutral-red granules described above can no longer be recognized. This phenomenon is surely no staining of true cytoplasmic granulations, but is merely caused by the precipitating reaction between the basophilic substance and the stains (fig. 7).

Despite the fact that most of the primitive blood-cells in the hanging-drop preparations or in the slide smears presented a more or less yellowish tint, I can not deny that in a few others such coloring was hardly recognizable. Some of these may have been degenerating megaloblasts; it requires, however, more accurate tests to determine the nature of such cells.

DIFFERENTIATION OF ERYTHROBLASTS STAGE I.

The megaloblasts rapidly increase in number, not only by their continued transmission from the blood-islands but also by their own mitotic division in the circulating blood. At the stage when the embryo has 16 to 18 pairs of somites there appears a second type of red cells, which is naturally the direct descendant of the megaloblast. This new generation is the erythroblast stage I, which corresponds exactly to Engel's "Metrozyten der ersten Generation" and to Dantschakoff's primary erythroblast.

These erythroblasts are still spherical in shape, but no longer possess pseudopodia or amœboid activity, having acquired definite cell membranes. Their cytoplasm is still noticeably basophilic but at the same time has accumulated a considerable amount of hemoglobin, which is unmistakably demonstrable by any of the methods of hemoglobin staining. The centrosphere is usually observable as a clear round spot beside the nucleus. The latter has diminished in size, but its chromatin substance, on the contrary, has increased. This substance appears as a distinct, coarse network, while the nucleolus is always found in it, round and sharply outlined (fig. 6). Mitotic divisions are frequently observed (fig. 10).

In hanging-drop specimens or supravital slide smears, erythroblasts of this stage exhibit the same phenomenon with neutral red and janus green as has been described for megaloblasts, except that the neutral-red granules arrange themselves in rosettes (fig. 5) and the precipitation of basophilic substance suffers a gradual decrease. The precipitates appear around the nucleus at first in round form, later often in a spindle shape, and the clear space at the periphery of the cytoplasm becomes wider and wider (figs. 9, 11).

DIFFERENTIATION OF ERYTHROBLASTS STAGE II.

Erythroblasts stage I constitute the main part of the blood-cells of the embryo from the end of the second day until the fourth day of incubation, continuing their mitotic division in the circulating blood. About the fifth day of incubation they suddenly give rise to a third generation of red cells—erythroblasts stage II. These cells are slightly larger than the mature red cells and now present the form of an oval or elongated oval disk. Their membranes are so compact that in cover-slip smears stained with eosin-methylene blue, rupture of the cell membrane and cytoplasm often takes place, leaving sharp, wedge-shaped clefts (fig. 14). Such ruptures have never been observed in any of the blood-cells except erythroblasts of this stage and erythrocytes. The basophilic substance in the cytoplasm has greatly decreased and the centrosphere has disappeared. The nucleus is strikingly reduced in size and its chromatin is arranged in a fine network, sometimes in radiating form. The nucleolus has entirely vanished (fig. 14).

In the neutral-red slide the granules are grouped in one spot or scattered irregularly throughout the cytoplasm or around the nucleus, but the rosettes are no longer to be found. The precipitates of the basophilic substance are greatly reduced in number and segregated in the immediate vicinity of the nucleus. Later, however, on the sixth day of incubation, they are found distributed throughout the cytoplasm in the form of a reticulum; we have here the so-called reticulated red cells (fig. 12). This reticulum can be produced by both neutral red and janus green.

Erythroblasts of stage II seem to coincide with Engel's "Metrocyten der zweite Generation" and Dantschakoff's definitive erythroblasts. According to the latter, they are derived not only from the primitive erythroblasts but also, and for the greater part, from the primitive blood-cells. I find, however, vigorous mitosis of erythroblasts stage I and at the same time every transitional form between them and erythroblasts of stage II in the blood of chicks on the fifth day of incubation. Erythroblasts stage II, therefore, may be considered as direct offsprings of erythroblasts stage I. Dantschakoff seems to have been misled by the fact that on the fifth day of incubation there is usually found a slight but definite increase in the number of megaloblasts. This fact, however, is not enough to explain the appearance of so great a number of erythroblasts stage II as to cover 21 to 92 per cent of all blood-cells (Tables 1 and 2). It is my opinion, as will be mentioned later, that such megaloblasts are consumed in the formation of the thrombocytes, but not of the erythroblasts stage II or Dantschakoff's "definitive erythroblasts."

DIFFERENTIATION OF NUCLEATED ERYTHROCYTES.

In the course of the seventh and eighth day of incubation, the erythroblasts stage II are gradually replaced by young nucleated erythrocytes. In the latter the basophilic or reticular substance has all disappeared, but at first a few neutral-red granules are still demonstrable, grouped or scattered in the cytoplasm (figs. 13, 15). After about the tenth day of incubation a small part of the young nucleated erythrocytes lose their neutral-red granules; most of them, however, still show from one to three, the peculiar movements of which were sometimes observed.

On the seventeenth day of incubation the typical mature nucleated erythrocytes make their appearance. Their nuclei are small, elongated oval in shape, and somewhat pycnotic, with fine zigzag outlines (fig. 16). The chromatin network has become denser, owing probably to the reduction in the size of the nucleus. In the fixed smears the cytoplasm is stained a beautiful red color without any trace of basophilic substance, but in the supravital slide many of the cells still exhibit one or two red granules.

It is noteworthy that there are a few spindle-shaped red cells in the blood of chicks, not only in embryonic life but also after hatching. These red cells ordinarily vary from medium size to exceedingly small, sometimes with one end pointed and the other rounded, sometimes with both ends pointed. Such red cells have appeared by the time the embryo has 22 to 29 somites, that is to say, from the stage of early erythroblasts; at this stage they are usually pointed at one end and rounded at the other (figs. 17, 18). As an evidence that such spindle-shaped red cells are by no means to be considered as artificial products, one finds them in the circulating blood within the vessels of the area pellucida. Their presence in the blood of lower vertebrates was first described by Löwit and was later emphasized by Knoll; the latter found them not only in most of the adults of cold-blooded vertebrates, but also in the blood of larvæ of *Rana esculenta*, *Pelobates fuscus*, *Salamandra maculata*, and of trout embryos.

The simple fact that a small part of red cells are spindle-shaped is important in so far that it has been, I believe, the cause of many confused and divergent views on the nature of thrombocytes or spindle-cells in the blood of fish, amphibia, reptilia, and birds. I shall discuss this problem in detail under the next caption.

DEVELOPMENT OF THE THROMBOCYTES.

When the chick has about 20 to 30 somites, a part of the megaloblasts occasionally give rise to elements of a new type, which are considered as transitional forms destined to differentiate further into the so-called thrombocytes. These transitional cells, or thromboblasts, are found to be almost constant elements of the circulating blood on the third day of incubation. They have diminished much in size, chiefly by losing a mass of cytoplasm, and their shapes are quite varied by the presence of psuedopodium-like processes. In stained cover-slip smears they are most frequently stelliform, with several short processes (figs. 29, 30). The cytoplasm is gradually being deprived of its basophilic substance and hemoglobin; it now shows a slight greenish color in the plain slide smears. The nucleus has also become small and stains uniformly with the Wright method, but usually more deeply than that of the megaloblast, and the chromatin figure is entirely obscured. At an early stage of thromboblast formation the nucleolus was occasionally found, but later no trace of it could be discerned. With vital methods these cells still exhibit tiny neutral-red granules or mitochondria around the nucleus, but by degrees these decrease in number.

In the course of the latter half of the third, or even on the fourth day of incubation, the thromboblasts further give rise to cells of mature thrombocyte type. These are the smallest of all blood-cells appearing after the fourth day of incubation, with the exception of some of the spindle-shaped red cells. In the living specimens they are oval or spindle-shaped (fig. 20), but in my fixed cover-slip smears only a few were observed to have kept their typical form (fig. 21), the others showing various indefinite shapes, with zigzag outlines, owing to the marked vulnerability of the cytoplasm (figs. 26, 27). Moreover, the cytoplasm has the particular property of fusing with that of cells of the same species. They sometimes form small clumps, consisting of 2 or 3 cells, but more often large clumps of 20 to 30 (fig. 22). In the early stage the thromboblasts can be seen to join these groups, but the megaloblasts do not appear to have as yet this property. It is of interest to note that even in the hanging-drop specimens of the area vasculosa, taken from the embryos on the fifth to the seventh day of incubation, I often observed the formation of large clumps of thrombocytes within the lumina of the blood-vessels. Owing to their striking cohesiveness, these cells can easily be distinguished from all other kinds of blood-cells.

The specific, basophilic substance and the hemoglobin have now both completely disappeared from the cytoplasm, though it still shows an affinity for both acidic and basic dyes in fixed smears. The former seems to be more manifest, at least during the first 10 days of incubation, but later, and even after hatching, the latter is usually predominant (figs. 22, 28). The eosinophilia of the cytoplasm of these cells has been well demonstrated by Engel, Dantschakoff, and others. Engel has shown in his illustration (1895, Taf. 17, fig. 4) a clump of thrombocytes with their cytoplasm stained an intense red color by hematoxylin-eosin staining, although he misinterpreted them as lymphocytes. Dantschakoff mentioned, too, the staining of their cytoplasm a violet or even rose-red tint with eosin-azur. In spite of such conspicuous affinity for eosin, I could find no trace of hemoglobin in these cells by observation of the living specimens. In well-fixed smears, one sees clear spaces surrounding the nucleus. These are called perinuclear spaces and are one of the characteristics of thrombocytes. In the final days of incubation and after hatching, one or two azur granules are often found in the cytoplasm (fig. 23). With supravital methods some of these cells showed one or two tiny neutral-red granules (fig. 20) or mitochondria, though many no longer exhibited them. Probably without any relation to such granulations, one or two dark refractive granules were occasionally met with in the cytoplasm.

Corresponding to the cell-body, the nucleus also is small and stains deeply blue in fixed smears, but often showing several coarse chromatin particles arranged in somewhat rotary form, resembling the nucleus of the plasma cells in adult animals. I did not find in a single case, however, a nucleolus or the much discussed Dekhuyzen's mitochrome.

From a survey of these findings, I have come to the conclusion that the thrombocytes are descendants of the megaloblasts but contain no hemoglobin, and that they play an important rôle in the formation of thrombosis. As is well known, the mode of differentiation of thrombocytes has hitherto been a most enigmatic problem, and one can not look back upon the history of its study without wondering at the very complicated views which have been set forth by various observers. Von Recklinghausen was probably the first to describe thrombocytes in the blood of the frog; then Ranvier, Golubew, Vulpian, Stricker, Hayem, and Macallum reported them in the blood of cold-blooded vertebrates as spindle-cells or fusiform corpuscles. Later they were summarized by Dekhuyzen under the term thrombocytes. Such cells in the blood of chicks, too, have been described by Bizzozero and Torre, Eberth, Dekhuyzen, Dantschakoff, Heinz, Kasarinoff, Nagai, and others. The views of all these authors concerning the development of thrombocytes may be classified under the following six concepts:

1. Thrombocytes are derived from lymphocytes or lymphoid cells (Neumann, Heinz, Eberhardt, Dantschakoff, Pappenheim, Werzberg).
2. Thrombocytes are mere transitory forms of white cells or lymphocytes (Riess, Löwit, Hlava), or even of red cells (Löwit).
3. Thrombocytes are damaged or degenerated red cells (Macallum, Schklarewsky, Eisen).
4. Thrombocytes are capable of giving rise to or of developing further into red cells (von Recklinghausen, Vulpian, Hayem, Marquis, Neumann, Heinz). This view has been especially emphasized by Hayem, who considered these cells as being of hemoblastic nature.
5. Thrombocytes are a type of giant cell (Rawitz and Wright).
6. Thrombocytes are derived from the endothelium (Golubew, Zahn, Ranvier, Marquis).

For the sake of simplicity, I shall begin the discussion of these concepts with the discrimination of thrombocytes, spindle-shaped red cells, and lymphocytes.

As I have already indicated under the foregoing heading, there are to be found nearly constantly in the blood of chick embryos, from the stage of 22 somites, a small number of red spindle-shaped cells, down to 7 on the day after hatching. They may be either pointed at one end and rounded at the other or pointed at both ends. Sometimes they present a much more typical spindle form than the oval-shaped thrombocytes in the fixed smears; but if one observes them closely, one can, without any difficulty, distinguish them from thrombocytes by their definite sharp cell membranes, characteristic form (one or both ends pointed), inequality of size, definite amount of hemoglobin or, in some cases, of basophilic substance which can be precipitated by vital stains, absence of perinuclear spaces, appearance of nucleus, and finally their inability to fuse. The spindle-shaped red cells and the thrombocytes are, therefore, entirely separate species, both from a physiological and a morphological point of view, except that both may present a spindle form. Strictly speaking, the thrombocytes in well-fixed smears of chick blood are oval rather than spindle-shaped. Dantschakoff likewise could find no true spindle-shaped thrombocytes in the bone-marrow of the chick embryo, but only those with both ends rounded. She referred to the frequent occurrence in cover-slip specimens of spindle thrombocytes with pointed ends, but these she considered as artificial products. Although her description differs slightly from my own, we are probably in accord regarding the view that thrombocytes in the blood of chick embryos do not present typical spindles with pointed ends.

It is quite obvious that the presence of spindle-shaped red cells in both embryonic and post-embryonic blood, coupled with the affinity shown by the cytoplasm of thrombocytes for eosin, is apt to mislead researchers who use the old terminology, *spindle-cell*, into the idea that thrombocytes can give rise to red cells, or that they are themselves damaged or degenerating red cells. Of course, this second view has been due partly to the marked vulnerability of thrombocytes.

In the second place, thrombocytes have often been taken for lymphocytes (Engel and many others who have described lymphocytes in the embryonic blood of chicks) or have been misinterpreted as mere transitory forms of white cells or lymphocytes (Riess, Löwit, Hlava). In my opinion, the thrombocytes have no similarity to or relationship with typical lymphocytes (here I do not speak of the primitive lymphoid cells). (1) Thrombocytes have a marked tendency to fuse and form cell clumps, while the lymphocytes lack this property; (2) thrombocytes have very delicate, vulnerable cell membranes, while lymphocytes present rather definite cell outlines; (3) the amœboid movements seem to be more vigorous in lymphocytes, while they are rarely seen in thrombocytes; it has even been denied by some authors (Hayem, Eberth, Neumann, Meves) that the thrombocytes are capable of such activity, although others (Stricker, Deetjen, Dekhuysen, Cajal, Riess) have recognized it in thrombocytes found in the blood of amphibia; (4) the cytoplasm of thrombocytes invariably shows more or less acidophilia; it often stains intensely red with eosin (Engel, Dantschakoff, Eberhardt, Werzberg), while the cytoplasm of lymphocytes is almost purely basophilic. In accord with the view of Bizzozero, Aynaud, Riess, Le Soud, and Painze, and opposed to that of Hayem, Heinz, and others, I conclude that in spite of such affinity for eosin, the thrombocytes contain no hemoglobin. Furthermore, it is noteworthy that there are no lymphocytes in the blood of chick embryos during the first 8 days of incubation, at least; the youngest embryo in which I found typical lymphocytes in the blood was of 17 days' incubation.

The above descriptions show that neither the mature red cells nor the lymphocytes have any relationship with the thrombocytes. The two concepts, therefore, that the thrombocytes are derived from lymphocytes and that they are temporary forms of lymphocytes are most probably incorrect. My view on the megaloblastic origin of thrombocytes, however, bears some similarity to those of Dantschakoff, Pappenheim and Werzberg, since they include under the term of *lymphocyte* the primitive blood-cell of Maximow or the lymphoidocyte of Pappenheim; while I believe, with Van der Stricht, Sabin, and many others, that the earliest blood-cells in the chick embryo are the megaloblasts.

It is hardly necessary to say that thrombocytes are in no way comparable to true giant cells. Such a misconception is due solely to the cohesiveness of these cells. However, Wright's idea that the thrombocytes precisely correspond to the giant cells or megakaryocytes of mammals is certainly worthy of attention, because in the blood of mammals, where the red cells are non-nucleated, the platelets, too, are non-nucleated; while on the other hand, in the blood of birds and other lower vertebrates the thrombocytes, as well as the red cells, are nucleated. Moreover, Wright has referred to the lack of megakaryocytes in the bone-marrow of the lower vertebrates.

As to the sixth concept, that endothelium can give rise to thrombocytes, it is fairly plain that there exists no direct relationship between the two, except that the megaloblasts, progenitors of thrombocytes, can be derived from the primitive endothelium.

Thus there is no question that the thrombocytes are independent cell elements in both embryonic and post-embryonic blood, as believed by Bizzozero, Eberth and Schimmelbusch, Dekhuyzen, Müller, Meves, Pappenheim, and others, and they they play a great rôle in thrombosis, as accepted by Bizzozero, Hayem, Eberth and Schimmelbusch, though this was opposed by Arnold, Schwalbe, Löwit, Neumann, Riess, and Helber. In conclusion I wish to suggest, in the matter of nomenclature, that such terms as *spindle-cells*, *fusiform corpuscles*, *hæmoblasts* (Hayem), and *plasmocytes* (Eisen) be discarded, both because they have given rise to such confusing views as those mentioned above and because of their being based upon misconceptions.

DEVELOPMENT OF THE PHAGOCYTIC CELLS.

Under this term I include clasmatocytes (Ranvier), adventitial cells (Marchand), "ruhende Wanderzelle" (Maximow), endothelial or wandering phagocytes (Maximow), macrophages (Evans), and histiocytes or histiocytoid cells (Kiyono).

One of the most important components of embryonic blood is the phagocytic cell. The youngest chick in which I found these cells had 12 pairs of somites. They are at first found only within the lumen of blood-vessels, but later also in the tissues. Most of them phagocytize 1 to 3 degenerating small cells or degenerating or apparently normal red cells; in some cases, however, they may engulf as many as 6 or 7 cells. Consequently, they vary greatly in size, from that of a megaloblast to twice the diameter of the endodermal wandering cells. If these cells are brought into contact with neutral red, the phagocytized cells or substances within their cytoplasm are promptly stained, and the cells can no longer be overlooked in the living specimens.

There can be recognized, at least before the circulation of the blood begins, two types of phagocytic cells in the lumen of the vessels. The first type is precisely the same size as the megaloblasts, possessing only blunt pseudopodia. In the cytoplasm one sees small refractive granules and occasionally droplets, but with neutral-red solution there are usually no red granules as long as the cells are still in good condition. The nucleus, with its round nucleolus, is often visible unless the phagocytized cells cover it. The cytoplasm, of course, commonly shows no hemoglobin, but in a few cases I could recognize a slight yellowish tint, although it is questionable whether these cells contained hemoglobin. The second type of phagocytes is the typical one, which is found in abundance in both the vessels and the tissues. They always have pseudopodia, long or short, pointed or blunt. With vital methods their cytoplasm exhibits many neutral-red granules and often mitochondria, scattered without order among the vacuoles. The nucleus is usually covered with such granules and vacuoles. In the cover-slip smears treated with Wright's stain, the cytoplasm seems to be less basophilic than that of phagocytes of the first type.

In a word, the phagocytes of the first type are characterized by their close similarity to the megaloblasts, while those of the second type represent the common, typical clasmatocytes. The former are found only in the earliest embryonic vessels, while the latter constitute one of the elements of the blood until the seventh or eighth day of incubation, and the more important elements of the tissues almost throughout embryonic and post-embryonic life. The oldest embryo in which I could find one of these cells in the blood was 12 days' incubation.

It is the primitive endothelium that can give rise to such intravascular phagocytic cells. The origin of phagocytic cells from endothelium was described by Sabin (1922, p. 54), but at that time she regarded them as identical with monocytes. Sabin, Doan, and Cunningham (1925) now believe that monocytes are a separate strain and do not come from endothelium. As one of the proofs for the theory that endothelium gives rise to the clasmatocyte type, I found 5 cases (embryos with 12 and 13 somites, 2 and 3 days old) in which the endothelial cells in their own position had engulfed degenerating cells. Maximow also found in the early embryonic blood of mammals phagocytes which he considers are derived from the endothelium of the area vasculosa.

The endothelial cells preserve their function of producing clasmatocytes until post-embryonic life, though only in limited regions of the blood-system, such as the Kupffer cells in the liver or the endothelium of the venous sinus of the spleen. Furthermore, in the frog the general venous endothelium has the function of producing clasmatocytes, as has been shown by Herzog in the capillaries of the tongue.

In addition to such intravascular formation of clasmatocytes, their production can be seen in the extravascular tissues. I found in 5 cases (embryos with 18 to 21 somites, third, fourth, and sixth days of incubation) that the mesenchymal cells in the adventitial position of capillary vessels were about to become free, showing a few neutral-red granules and vacuoles. These cells are, however, interpreted by Sabin as endothelial.

After the fifth day of incubation one sees numerous clasmatocytes in the tissues of the allantois and chorion. It seems quite probable that they are derived from the mesenchymal cells. The typical mesenchymal cells have many mitochondria but no neutral-red granules. As soon as they begin to isolate, there appear, one by one, several granules stained with neutral red and, later, also vacuoles. At the same time the cell-body becomes round by the retraction of its processes, finally separating from the syncytial plexus of mesenchyme. The mitochondria seem to be preserved for a long while, and if a piece of allantois or chorion is put in a solution of janus green for 1 to 2 hours, one always finds blue granules appearing among vacuoles in clasmatocytes. I believe, therefore, that the mesenchymal cells give rise to extravascular clasmatocytes, as accepted by Maximow, Dantschakoff, and others.

Another question still remains, i. e., in regard to the formation of intravascular phagocytic cells of the first type described above in the earliest blood-vessels. There are at least three possible explanations for their similarity to the megaloblasts: (1) that the endothelial phagocytes merely bear a close resemblance to the megaloblasts, both being direct derivatives of endothelium; (2) that the megaloblasts or primitive blood-cells can differentiate into phagocytes; (3) that the megaloblasts themselves are able to phagocytize. I have found no evidence in favor of any of these possibilities; I might add, however, that Dantschakoff has illustrated a primitive blood-cell that had engulfed a degenerating cell as "phagozytierende Lymphozyt" (1908, Taf. 28, fig. 5), and such an idea has also been accepted by Kiyono. Naturally, further investigations are needed before this important question can be decided.

I wish to describe briefly the adventitial cell of the blood-vessels, since it is somewhat concerned with the differentiation of the clasmatocytes and of the myeloblasts. The cell layers in the adventitial position were occasionally observed first at the stage of 13 to 14 somites. It is usually on the third day of incubation that one sees in various regions continuous layers of primitive adventitia. Later, fourth to sixth day of incubation, one finds thick layers of cells forming the walls of large vessels, while, on the other hand, numerous vessels still consist of a simple layer of endothelium.

These adventitial layers of blood-vessels make their appearance, as a rule, later than the establishment of capillary endothelium, not by the proliferation of endothelium, but positively through the proliferation of mesenchymal cells in the so-called substance islands. Moreover, these cells of the adventitial layers show much less affinity for neutral red than does the endothelium. Therefore, they should be interpreted neither as the outer row of reduplicated endothelium nor as Marchand's adventitial cells, but as mesenchymal cells that have proliferated just outside of the endothelial borders. It is such mesenchymal cells, in their adventitial position or in the meshes of the networks of blood-vessels, that further differentiate into the extravascular clasmatocytes and also into myeloblasts.

DIFFERENTIATION OF THE WHITE CELLS.

EOSINOPHILIC LEUCOCYTES.

In the latter half of the third day or on the fourth day of incubation, the mesenchymal cells around the blood-vessels begin to separate from the syncytial plexus. They gradually lose their processes and become free as round, extravascular myeloblasts. Such cells are never comparable to the intravascular primitive blood-cells or megaloblasts, since their cytoplasm is much clearer and contains only a few minute refractive granules, without, of course, the slightest shadow of hemoglobin. Moreover, they show no neutral-red granules, while the megaloblasts show not only these granules, but also tremendous precipitates of basophilic substance when stained with neutral red or janus green. The myeloblasts, however, exhibit many tiny mitochondria distributed throughout the cytoplasm.

In such myeloblasts small and slightly refractive neutral-red granules appear promptly, increase in number, and arrange themselves in beautiful rosettes, while the mitochondria diminish in number and finally are found only in the periphery of the cytoplasm. At the same time the nucleus, which was perfectly round at the start, becomes semispherical, then kidney-shaped, and at last horseshoe-shaped. Now we see the typical myelocytes, which are always found in the area pellucida of embryos on the fourth or fifth day of incubation. Later their round granules become elongated fusiform. The wandering of these cells through the endothelial walls into the lumen of the blood-vessel takes place first on the fifth day, but they are as yet but seldom met with in the circulating blood. For example, I found in one case only 0.1 per cent of them as against all other blood-cell types. From the seventh day of incubation there is a rapid increase in their absolute number.

The first leucocytes in the blood of chick embryos, therefore, are the polymorphonuclear or polynuclear cells with tiny spindle-shaped granules, stainable with eosin in the fixed smears; they are the so-called pseudo-eosinophilic leucocytes, which are generally believed to coincide with the polymorphonuclear neutrophilic leucocytes in the blood of mammals. In the vital slides they move with measurable rapidity, and the nuclei, often two in number, are clearly seen to have divided in the cytoplasm. It is noteworthy that several round neutral-red granules appear in the cytoplasm, often near the nuclei, while the characteristic spindle-shaped granules remain unstained for a long time; afterward they stain faintly with both neutral red and janus green. The same phenomenon can be observed in the neutrophilic leucocytes in mammalian blood (rabbits, etc.); in the neutral-red slide specimens such cells very promptly show several neutral-red granules, while many other characteristic refractive granules can be stained after 1, 2, or 3 hours.

The true eosinophilic leucocytes with round refractive granules are found, as a rule, only in the last days of incubation, but in exceptional cases I found some leucocytes with round granules on the seventh day of incubation. They seem to be produced mainly in the spleen. In the vital slides they, also, wandered quickly and exhibited several small neutral-red granules, while the characteristic round granules were not stained for a long while. They showed nothing different from the pseudo-eosinophilic leucocytes, except that their granules were round instead of spindle-shaped. I could not tell, therefore, whether they were merely immature forms of pseudo-eosinophilic leucocytes or the true eosinophilic leucocytes. In one case these two types of cells were present in the vital slides in the proportion of 35 to 2 respectively. In fixed smears of the same blood I was unable to identify them, because in these early days the granules of eosinophilic leucocytes are so small that it is beyond the power of the microscope to show their shape in fixed smears.

BASOPHILIC LEUCOCYTES.

These cells were first noted in the chick's blood on the fourteenth day of incubation, and again on the seventeenth. In that period there are always to be found basophilic cells in the spleen, but they may also be produced by the thymus, as has been described by Dantschakoff.

LYMPHOCYTES.

Typical lymphocytes were found first in the blood of a 17-day old embryo. Some were observed as early as the fourteenth and fifteenth day, but these were not quite typical. It is most striking that the blood of embryo chicks, except in the last few days of the incubation period, contains no lymphocytes, although numerous investigators have described them at this time (Engel, Dantschakoff, and many others). It is quite probable that the thrombocytes have been mistaken for lymphocytes.

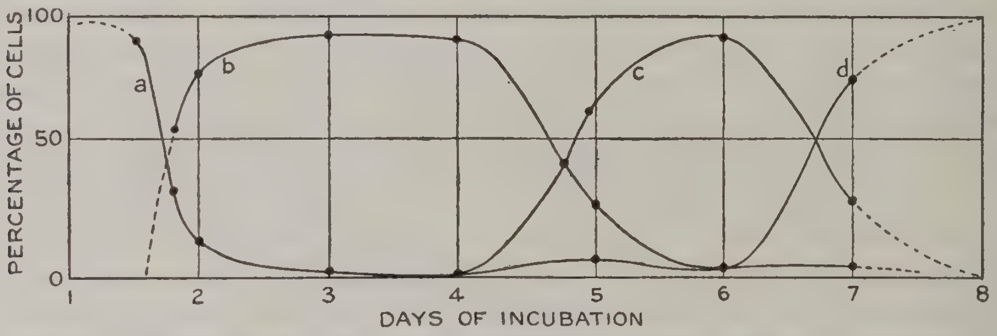
MONOCYTES.

Through the whole embryonic period I could find no typical monocytes in the circulating blood, though after the seventeenth day of incubation atypical cells were occasionally observed.

I did not make any extensive study of hematogenesis in the hemitopoaetic organs, but merely studied the spleen in embryos 14 to 19 days old, with vital methods. There were always observed vigorous proliferation of pseudo-eosinophilic and true eosinophilic myelocytes and leucocytes, basophilic leucocytes, and a relatively few lymphocytes or monocytes.

GENERAL DATA.

Table 1 gives a precise account of the numerical relations of each species of blood-cells. All figures in the table have been obtained by counting of cover-slip smears stained with Wright's eosin-methylene blue. In every case I counted the cells in vital slides of the same blood, but omitted the figures from the table because of the somewhat inconstant results, due to the difficulty of exact classification of cells in these specimens.



TEXT-FIG. 1.—Graph prepared from Tables 1 and 3, in order to give a fair idea of rise and fall of megaloblasts, erythroblasts stage I and II, and nucleated erythrocytes, from second to seventh day of incubation. Day of incubation: *a*, megaloblast; *b*, erythroblast stage I; *c*, erythroblast stage II; *d*, nucleated erythrocytes.

From table 1 it can be readily seen (1) that erythroblasts stage II are produced at the cost of erythroblasts stage I, but never of megaloblasts or primitive blood-cells, as was believed by Dantschakoff and Maximow, for there is a great gap between the percentage of erythroblasts stage II and of megaloblasts, while, on the other hand, the predominance of erythroblasts stage I is closely followed by that of erythroblasts stage II; (2) that from the third day of incubation there is a correlative relationship between the percentages of megaloblasts and those of thromboblasts or thrombocytes. Although one sees a slight increase of megaloblasts on the fifth day of incubation, they are certainly consumed in the formation of thromboblasts and thrombocytes, but not of erythroblasts stage II, because such inconspicuous increase of megaloblasts (6 to 8 per cent) can never account for the rapid multiplication of erythroblasts stage II (21 to 92 per cent).

It may be mentioned that one ought to be careful not to judge at once the disappearance or decrease in the absolute number of any species of cells from the percentage given in Table 1, for it is often only apparent and is caused by the vigor-

Table 1.—*Percentage of each species of blood-cells drawn from the vessels in the area pellucida of chick embryos during the first 7 days of incubation.*

Number of embryos.	Ages of embryos.	Endodermal wandering cells.	Degenerating small cells.	Megaloblasts.	Intermediate forms.	Erythroblasts stage I.	Intermediate forms.	Erythroblasts stage II, and intermediate forms.	Young nucleated erythrocytes.	Thromboblasts.	Thrombocytes.	Clasmatocytes.	Eosinophilic leucocytes.	Damaged or degenerated red cells.
60	14 som.	1.0	1.0	95.9	2.1	......	......	......	......	......	......	......	......	(4.0)
58	18 som.	......	0.7	23.1	......	64.9	......	......	......	1.3?	......	......	......	(23.0)
65	20–21 som.	......	1.7	43.4	......	50.2	......	......	......	4.7?	......	......	......	(0.3)
63	25 som.	......	......	20.0	6.5	73.5	......	......	......	......	......	......	......	(0.7)
56	29–30 som.	......	......	3.6	1.5	91.1	......	......	......	3.8	......	......	......	(21.3)
53	3 days	......	......	1.1	......	93.8	......	......	......	2.8	1.7	0.6	......	(11.5)
55	Do.	......	......	5.5	......	83.1	......	......	......	6.7	3.8	0.9	......	(41.0)
64	Do.	......	......	......	......	98.6	......	......	......	0.7	......	0.7	......	(18.4)
66	Do.	......	......	1.1	......	95.6	......	......	......	1.4	1.1	0.8	......	(9.7)
67	Do.	......	......	0.3	......	97.4	......	......	......	0.6	1.7	......	......	(0.3)
68	4 days	......	......	1.1	......	92.9	......	......	......	1.4	4.1	0.5	......	(12.0)
86	Do.	......	......	1.3	......	86.1	7.3	1.1	......	2.4	1.8	......	......	(6.5)
71	5 days	......	......	6.3	......	65.0	......	21.2	......	2.5	5.0	......	......	(20.0)
121	Do.	......	......	8.0	......	5.2	......	75.9	......	3.8	7.1	......	......	(4.6)
72	Do.	......	......	0.4	......	2.7	......	92.6	......	0.4	2.5	1.3	0.1	(3.0)
74	6 days	......	......	0.8	......	2.1	......	93.2	......	0.6	2.9	0.4	......	(4.2)
76	7 days	......	......	......	......	0.9	......	33.3	59.8	0.4	5.6	......	......	(4.5)
75	Do.	......	......	......	......	1.1	......	22.1	74.6	0.5	1.7	......	......	(2.3)
50	Do.	......	......	......	......	0.6	......	9.6	88.7	......	0.9	......	0.2	(1.8)

Table 2.—*Percentage of blood-cells taken from chick embryos on seventh to fourteenth day of incubation, exclusive of erythroblasts stage II and erythrocytes.*

Number of embryos.	Ages of embryos.	Megaloblasts.	Erythroblasts stage I.	Thromboblasts.	Thrombocytes.	Clasmatocytes.	Pseudo-eosinophilic leucocytes.	Basophilic leucocytes.
75	7 days	1.3	5.5	5.3	85.7	0.7	1.5	....
76	Do.	....	5.5	7.5	84.0	....	3.0	....
77	Do.	0.5	3.3	3.5	88.0	1.0	3.7	....
79	10 days	2.0	10.0	....	83.0	....	5.0	....
81	14 days	3.0	9.0	....	67.0	....	20.0	1.0

Table 3.—*Average percentages of red cells at each day of the first seven days of incubation, calculated from Table 1.*

Ages of embryos.	Megaloblasts.	Erythroblasts stage I.	Erythroblasts stage II and intermediate forms.	Young nucleated erythrocytes.	All other cells.
1½ days..........	96.0	....	....	....	4.0
1¾ days..........	33.5	57.5	....	....	9.0
2 days...........	12.0	82.5	....	....	5.5
3 days...........	1.5	93.5	....	....	5.0
4 days...........	1.2	89.5	0.5	....	8.8
5 days...........	5.0	24.5	65.0	....	5.5
6 days...........	0.8	2.0	93.0	....	4.2
7 days...........	....	1.2	21.5	74.5	2.8

ous proliferation of nucleated erythrocytes or erythroblasts stage II. For example, the megaloblasts do not disappear during embryonic life, although they have not been figured from the seventh day of incubation; and the thrombocytes or pseudo-eosinophilic leucocytes steadily increase in number, despite their decreasing or quite variable percentages. This may be plainly seen in Table 2, in which the erythroblasts stage II and nucleated erythrocytes have been excluded from the reckoning. It can also be seen from this table that from the seventh to the fourteenth day of incubation the thrombocytes are the leading non-hemoglobin bearing blood-cells; the pseudo-eosinophilic leucocytes also rapidly increase in number during this period.

SUMMARY.

The results of this study may be summarized as follows:

1. Red and white cells are derived from different stem-cells. In the area pellucida of chick embryos the primitive red cells or megaloblasts develop from blood-islands and primitive endothelium, while the myeloblasts or progenitors of the pseudo-eosinophilic leucocytes originate directly from the extravascular mesenchymal cells, the mature leucocytes then migrating into the blood-vessels through the capillary walls. In exceptional cases (embryos of the seventh day of incubation) eosinophilic leucocytes with round granules were found in the circulating blood. These showed vigorous ameboid movements in the slide specimen and had precisely the same appearance as the pseudo-eosinophilic leucocytes, except that they contained round granules instead of rods. Usually, true eosinophilic leucocytes make their first appearance in the blood during the last few days of incubation.

2. The basophilic leucocytes are first found in embryonic blood on the fourteenth day of incubation. At this stage numerous myeloblasts, eosinophilic myelocytes and leucocytes, and basophilic leucocytes can be found in the spleen.

3. It is the megaloblasts that give rise to the thromboblasts and thrombocytes. Most of the authors have probably been misled into such conflicting views as mentioned in the introduction by the existence of true spindle-shaped red cells in both embryonic and post-embryonic blood, the lack of lymphocytes in the blood of the chick during the first half of the incubation period, and such cytoplasmic properties of the thrombocytes as acidophilia, vulnerability, and tendency to fuse. A number of observers have misinterpreted the thrombocytes as lymphocytes, but typical lymphocytes appear in the blood only in the final days of incubation. They seem to be produced in the spleen and in other lymphatic tissues.

4. The intravascular phagocytic cells owe their origin to the primitive endothelium, while the extravascular ones originate most probably from the mesenchymal cells.

5. It is noteworthy that in the primitive blood-vessels of the area pellucida there are recognizable two types of phagocytic cells: one characterized by its close similarity to the primitive blood-cells or megaloblasts, the other presenting the typical appearance of normal clasmatocytes in the tissues. These phagocytic cells

are the normal elements of the circulating blood of embryos during the first 8 days, disappearing promptly thereafter. Nevertheless they are of common occurrence in the tissues of the embryonic body, allantois, chorion, and amnion through the whole period of incubation.

6. The monocytes or Ehrlich's large mononuclears and transitional forms, though not typical, are found in the blood only toward the end of incubation.

In conclusion I wish to express my indebtedness to Dr. F. R. Sabin and Dr. R. S. Cunningham for friendly guidance and advice during the progress of this investigation; also to Mr. James F. Didusch for his assistance in the preparation of the illustrations.

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DESCRIPTION OF FIGURES.

All cells on this plate are from the blood of chick embryos with the exception of one, which is a blood-cell drawn from a chick on the second day after hatching. About half of the figures were drawn with the camera lucida; the other half are free hand.

Plate 1.

Fig. 1. Mesenchymal cells from the subcutaneous tissue of a chick embryo with 24 somites. Supravital staining with janus green.

Fig. 2. A blood-island from the posterior part of the area pellucida of an embryo with 15 somites. Supravital staining with neutral red. (Hanging-drop specimen.)

Fig. 3. Megaloblast from an embryo with 23 somites. Supravital staining with neutral-red and janus green. (Slide smear.)

Fig. 4. Megaloblast from blood drawn from the area opaca of an embryo with 9 somites. Stained with Wright's eosin-methylene blue. (Cover-slip smear.)

Fig. 5. Erythroblast stage I, with a rosette, from a 4-day embryo. Supravital staining with neutral red. (Slide smear.)

Fig. 6. Erythroblast stage I, from the blood of a 4-day embryo. Wright's stain. (Cover-slip smear.) Figures 5 and 6 represent an older stage than figures 7 and 8, and should follow them.

Fig. 7. Megaloblast with precipitated basophilic substance. From the same slide as figure 3. This is a younger stage than figures 5 and 6 and should have preceded them.

Fig. 8. Megaloblast from blood of an embryo of 14 somites. Wright's stain. (Cover-slip smear.) This, like figure 7, is a younger stage than the cells shown in figures 5 and 6.

Fig. 9. Erythroblast stage I, with precipitated basophilic substance. From an embryo with 23 somites. Supravital staining with neutral red. (Slide smear.)

Fig. 10. Erythroblast showing mitosis; from the blood of an embryo of 18 somites. Wright's stain. (Cover-slip smear.)

Fig. 11. Erythroblast stage I with precipitated basophilic substance; from a 4-day old embryo. Supravital staining with neutral red. (Slide smear.)

Fig. 12. Erythroblast stage II on a slide of double stains. From a 6-day old embryo. It exhibits reticular substance stained with neutral red.

Fig. 13. Young nucleated erythrocyte from the blood of a 9-day old embryo. Supravital staining with neutral red. (Slide smear.)

Fig. 14. Erythroblast stage II, showing a wedge-shaped cleft; from the blood of a 5-day old embryo. Wright's stain. (Cover-slip specimen.)

Fig. 15. Nucleated erythrocyte from the blood of a 10-day old embryo. Wright's stain. (Cover-slip specimen.)

Fig. 16. Mature nucleated erythrocyte from the blood of a 17-day old embryo. Wright's stain. (Cover-slip smear.)

Fig. 17. Spindle-shaped erythrocyte from the blood of a 12-day old embryo. Supravital staining with neutral red. (Slide smear.)

Fig. 18. Spindle-shaped red cell corresponding to the nucleated erythrocyte. From the blood of a 15-day old embryo.

Fig. 19. Transitional forms between megaloblasts and thromboblasts. From a 2-day old embryo. Supravital staining with neutral red. (Slide smear.)

Fig. 20. Thrombocyte from the blood of a 5-day old chick embryo. Supravital staining with neutral red and janus green. (Slide smear.)

Fig. 21. Thrombocyte from the blood of a 6-day old embryo. Wright's stain. (Cover-slip smear.)

Fig. 22. A small clump of thrombocytes, showing partly a bluish tint (basophilic) and partly a reddish tint (acidophilic). From blood of a 6-day old embryo. Wright's stain. (Cover-slip specimen.)

Fig. 23. A thrombocyte containing two azure granules. From the blood of a chick 2 days after hatching. Wright's stain. (Cover-slip smear.)

Figs. 24–30. This group of cells, representing various stages in the process of thrombocytogenesis, was found in a 5-day chick embryo. The specimen was stained with Wright's solution.

Figure 25, megaloblast, nearly typical but very small. Figures 29 and 30 represent thrombocytes still showing much resemblance to megaloblasts but furnished with fine processes. (Cf. with fig. 8.)

Figures 26 and 27 show cells considered to be rather young thrombocytes with fine processes (probably artificial products).

Figure 28, typical thrombocyte, with cytoplasm stained a reddish color with eosin.

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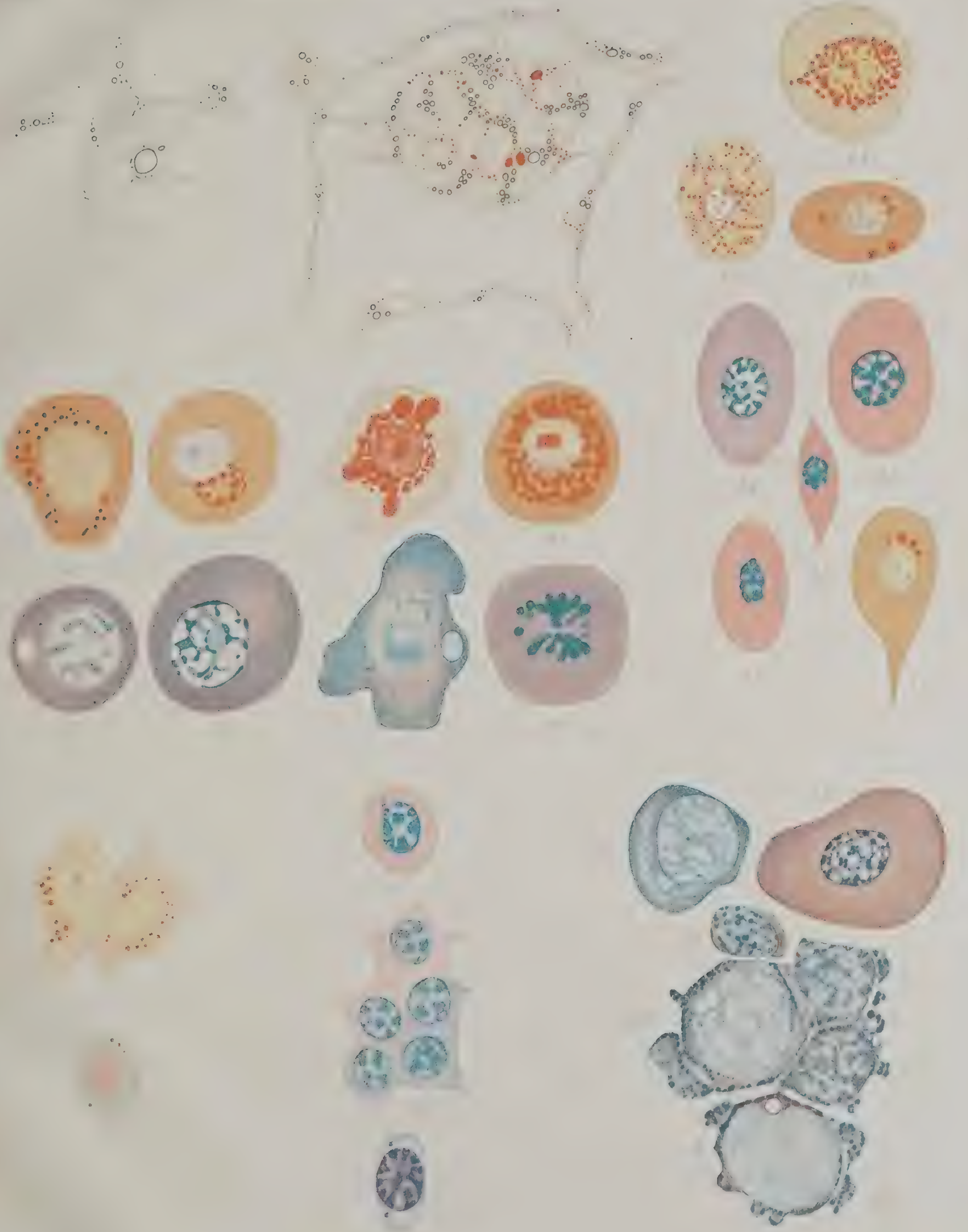
SUGIYAMA

PLATE 1



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